Biological and Medical Applications of Materials and Interfaces

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Multi-functional Nanobio Hybrid Material Composed of Ag@Bi\textsubscript{2}Se\textsubscript{3}/RNA Three-Way Junction/miRNA/Retinoic Acid for Neuroblastoma Differentiation

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Abstract

Nanoparticle-based cell differentiation therapy has attracted increasing research interest as it is a promising substitute for conventional cancer treatment methods. Here, the topological insulator bismuth selenide nanoparticle (Bi$_2$Se$_3$ NP) was core–shelled with silver (Ag@Bi$_2$Se$_3$) to represent remarkable biocompatibility and plasmonic features (ca. 2.3 times higher than those of Ag nanoparticle). Moreover, a newly developed RNA three-way junction structure (3WJ) was designed for the quad-functionalization of any type of nanoparticle and surface. One leg of the 3WJ was attached to the Ag@Bi$_2$Se$_3$, and the other leg harbored a cell-penetrating RNA and a fluorescence tag. The third leg was designed to inhibit microRNA-17 (miR-17) and to further release retinoic acid (RA). A new drug delivery mechanism was developed for the slow release of RA inside the cytosol based on the prerequisite inhibition of miR-17 using strand displacement strategy. In this paper, we report a simple methodology for resolving the hydrophobicity challenges of RA by its conjugation with a RNA strand (RA/R) through a stimulus-responsive cross-linker. The developed nanobiohybrid material could fully differentiate SH-SY5Y cancer cells into neurons and stop their growth in 6 days without requiring sequential treatments which has not been reported yet. Using surface-enhanced Raman spectroscopy technique, the RA delivery and the cell differentiation process were monitored nondestructively in real time. The fabricated nanobiohybrid material could open the new horizons in the fabrication of different diagnostic/therapeutic agents.

Keywords: silver Bi$_2$Se$_3$ core–shell nanoparticle, microRNA inhibition, drug delivery, cell differentiation therapy, SERS, multifunctional nanoparticle, RNA three-way junction, cancer
1. Introduction

Besides traditional chemo- and radio-therapy, differentiation therapy is an important therapeutic method resulting in irreversible changes in cancer cell phenotypes. It aims to i) resume the cell maturation process with less toxicity and ii) revert the cancer cells into normal cells without damaging the non-target cells\textsuperscript{1–4}. In spite of various treatment methods, neuroblastoma (NB) remains the most prevalent high-risk solid tumor in children, with a long-term survival rate of \( \sim 40\% \)\textsuperscript{5,6}. According to the literature, NB growth can be effectively hampered by differentiation\textsuperscript{7,8}. However, current and traditional NB differentiation methods, such as treatment with RA and/or brain-derived neurotrophic factor, require sequential drug treatments and removal of serum from media\textsuperscript{9–11}. These are inefficient methods because of the time-consuming (2 to 3 weeks) and labor-intensive procedures, as well as obstacles due to drug hydrophobicity. Considering the cytotoxic effect of RA, precise control over its dose and administration time is crucial to avoiding apoptosis induction and unwanted shear stresses\textsuperscript{12,13}. On the other hand it has been reported that down-regulation of microRNA-17 (miR-17) can induce NB differentiation and promote RA activity\textsuperscript{14}. Resolving the above-mentioned challenges and subsequent cooperation of RA induction and miR-17 inhibition might improve the yield of NB differentiation. However, the low transfection efficiency of the therapeutic RNAs (miRNAs and siRNAs) should be taken into account\textsuperscript{15–18}.

With the aim of improving the current cell differentiation strategies, various inorganic/organic nanostructures in the form of nanoscaffolds (nanopatterned surfaces) have been used. Despite their high throughput and cell imaging capability, these approaches are only in vitro. The proposed differentiation procedures are relatively long and require tedious media exchange, which is prone to cell loss followed by low efficiency\textsuperscript{19–21}. Additionally, the existing
nanoparticle-based cell differentiation methods are either prolonged or require complicated chemistry for the nanoparticle (NP) functionalization\textsuperscript{21,22} (for more references, refer to Table S1). Despite that, multi-functionalization of NPs while keeping enough intervals between the immobilized agents on the surface to allow them to accurately perform their tasks without additional hindrance effects is generally a big challenge in NP-based diagnosis and/or therapy\textsuperscript{23}. Up until now, no studies have achieved the controllable tri- or quad-functionalization of NPs except for the bi-functionalization methods\textsuperscript{24,25} and other uncontrollable procedures based the sophisticated and complicated chemistry\textsuperscript{26–28} (for more references, refer to Table S2).

Plasmonic NPs\textsuperscript{29} have shown great potential for noninvasive cell fate monitoring\textsuperscript{30,31} and cargo release tracking\textsuperscript{32,33} based on surface-enhanced Raman spectroscopy (SERS). One of the most exotic plasmonic materials is topological insulator (TI) Bi\textsubscript{2}Se\textsubscript{3}\textsuperscript{34}. The surface states of TI supply a two-dimensional (2D) Dirac structure from a bulk material at room temperature (RT) that is observed only in graphene at the atomic layers far below the RT\textsuperscript{35}. Furthermore, TIs are topologically protected\textsuperscript{36}; therefore, similar properties could be expected from two TIs with slightly different topologies. This can considerably alleviate the reproducibility and reliability issues of NP applications. However, the biomedical applications of TIs are still limited because of their hydrophobicity, surface functionalization challenges, and low biocompatibility\textsuperscript{37,38}.

To address the aforementioned challenges, the synthesized Bi\textsubscript{2}Se\textsubscript{3} NP was core–shelled with Ag (Ag@Bi\textsubscript{2}Se\textsubscript{3}, \textasciitilde50 nm) to increase its biocompatibility and to restore its peculiar plasmonic features (\textasciitilde2.3 times higher than those of silver nanoparticle (AgNP)). Compared with AgNP, Ag@Bi\textsubscript{2}Se\textsubscript{3} had more stable optical properties, which may be due to the topologically protective nature of Bi\textsubscript{2}Se\textsubscript{3}. To enhance its colloidal stability, the surface of the Ag@Bi\textsubscript{2}Se\textsubscript{3} NP was treated with polyvinylpyrrolidone (PVP) (denoted as Ag@Bi\textsubscript{2}Se\textsubscript{3}/PVP). We could develop an adjustable
quad-functionalization method using an ultra-stable RNA three-way junction (3WJ) structure\textsuperscript{39–41}. This structure was further modified to be conjugated onto the Ag@Bi\textsubscript{2}Se\textsubscript{3} NP (silver–thiol interaction) and to harbor a nominal “otter” cell-penetrating RNA (CPR)\textsuperscript{42}, a fluorescence tag, a miR-17 inhibitor moiety, and an RA-modified RNA structure (RA/R) to form the Ag@Bi\textsubscript{2}Se\textsubscript{3}/PVP-3WJ-RA/R final structure. Compared with that in our previous work\textsuperscript{43}, the number of functional groups on the Ag@Bi\textsubscript{2}Se\textsubscript{3} NP surface was considerably boosted, from \(\sim 74\) to \(\sim 1376\) molecules per NP. We could also simply resolve the RA hydrophobicity problem by its conjugation with an RNA strand through the disulfide-breakable cross-linker 3-(2-pyridyldithio)propionyl hydrazide (PDPH) which has not been reported elsewhere.

As well, a newly developed drug delivery system was introduced for the slow release of the RA inside the cytosol based on the prerequisite miR-17 inhibition using the strand displacement mechanism. The process was initiated at a complementary sequence (toehold) followed by subsequent migration of the pre-hybridized RA/R (Figure 1A). Migration of the RA/R caused removal of RA from close vicinity of NP surface, resulting in a significant SERS signal drop of the RA (Figure 1B). The disulfide bond between the RA and R was further broken inside the cytoplasm via the main disulfide-bond-breaking agent glutathione (GSH) in the tumor tissues\textsuperscript{44}. Since GSH has a net negative charge at physiological pH\textsuperscript{45}, having RA buried inside the negatively charged Ag@Bi\textsubscript{2}Se\textsubscript{3}/PVP-3WJ-RA/R prevented the GSH molecule from effectively reaching the RA before having the RA/R displaced by the miR-17 (Figure 1). The developed nanobiohybrid material could fully differentiate SH-SY5Y cells into neurons in 6 days without requiring post-treatment. Tracking the RA delivery and monitoring the cell differentiation process were carried out using the SERS technique in a nondestructive and real-time manner. In
addition, we could observe a cell-to-cell differentiation phenomenon in which the differentiated cells tended to seek and differentiate the closest non-differentiated cells.

2. Materials and methods

2.1. Chemicals and reagents

Bismuth(III) nitrate pentahydrate, PVP, triethanolamine, silver nitrate, hydrazine hydrate, triethylamine, dicyclohexylcarbodiimide, N-hydroxysuccinimide (NHS), sodium selenosulfate solution, trisodium citrate dehydrate (TCEP), reduced GSH, phosphate-buffered saline (PBS, pH 7.4), and 4-mercaptobenzoic acid (4-MBA) were supplied by Sigma-Aldrich (USA). C$_2$H$_6$O, H$_2$SO$_4$, acetone, diethyl ether, and dimethylformamide (DMF) were supplied by Daejung Chemical (ROK). The transmission electron microscopy (TEM) grids were obtained from Ted Pella Inc. (USA). The dithiothreitol was purchased from Pierce (USA). Lipofectamine® RNAiMAX reagent, Hoechst anti-MAP2 antibody, FITC-labeled secondary antibody, and LysoTracker Green DND-26 were supplied by Thermo Fisher Scientific. The DNA and RNA strands were chemically modified by 2′-Fluoro (2′F) and provided by Bioneer® (Korea). The size and shape of the NPs were measured via TEM (JEM-2100F, JEOL), which was done at an accelerating voltage of 200 kV. The Milli-Q system (Millipore, USA) was used for deionized (DI) water purification. The size distribution and surface charge of the nanobiohybrid materials were confirmed via dynamic light scattering (DLS) (ELSZ-1000, Otsuka). SERS analysis of the nanobiohybrid materials was done using confocal Raman spectroscopy (Ntegra Spectra, NT-MDT). Fluorescence imaging was performed using a confocal laser scanning microscope (CLSM, Zeiss Co., Germany). The optical absorbance of the NPs was measured using a UV–Vis spectrophotometer (Jasco V530; Easton, MD, USA) and a microplate reader (EL800, Biotek,
USA). An ultrahigh-resolution mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific) was used for the conjugation analysis. A LightCycler ® 2.0 instrument (Roche) was used for the qRT-PCR experiments.

2.2. Synthesis and characterization of the Ag@Bi$_2$Se$_3$ core–shell NPs

For the synthesis of Ag@Bi$_2$Se$_3$ core–shell nanostructures, Bi$_2$Se$_3$ was synthesized, and then Ag was grown onto the Bi$_2$Se$_3$. In a typical process, 0.819 g of bismuth nitrate was dissolved in 30 mL of distilled water inside a 250 mL round bottom flask. Subsequently, 20 mL of triethanol amine was injected into the solution under constant stirring to form a homogeneous solution. Next, a calculated amount of sodium selenosulfate solution was added. The solution pH (9–10) was adjusted using triethanol amine at 70°C for 4 hr. The entire reaction was conducted under N$_2$ atmosphere. The black precipitate was filtered and washed several times with distilled water and methanol to eliminate the impurities and by-products. The final product was dried in vacuum for 5 hr at 60°C. Afterward, 0.10 g of Bi$_2$Se$_3$ was dispersed in 20 mL of 1:1 water/DMF mixture. This was followed by the addition of 0.026 g AgNO$_3$ and 0.60 g of PVP (Mw: 10 kDa) in 10 mL of DI water and stirring for about 1 hr at 60°C. Hydrazine hydrate solution (2 mL) was rapidly added to the solution and stirred for about 1 hr. The precipitate was subsequently filtered and thoroughly rinsed with DI water and alcohol and finally dried in oven.

2.3. Cell culture

The NB cell line (SH-SY5Y) was supplied by ATCC (Manassas, VA, USA). The cells were cultured at 37°C in DMEM supplemented with 1 % antibiotics (streptomycin and penicillin) and 10 % heat-inactivated fetal bovine serum in a humid chamber in the presence of CO$_2$ (5 %). The
cells were cultured in TC-grade petri dishes, and the medium was replenished every 48 hr during incubation.

### 2.4. Oligonucleotide sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' =&gt; 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CUACCUGCUUCUGUAAGCACUUUGUUGCCAUGUAGUGGGAAGAGCUUGGCUUUUGUGCGAAAGCACCUAUGAUCACACUCC</td>
</tr>
<tr>
<td>Scrambled A</td>
<td>GACGUAAGCUUCUGUAAGCACUUUGUUGCCAUGUAGUGGGAAGAGCUUGGCUUUUGUGCGAAAGCACCUAUGAUCACACUCC</td>
</tr>
<tr>
<td>B</td>
<td>CCCACAUACUUUGUUGAUCC</td>
</tr>
<tr>
<td>B-TR</td>
<td>TR-CCCACAUACUUUGUUGAUCC</td>
</tr>
<tr>
<td>C</td>
<td>GGAUCAAUCAUGGCAA</td>
</tr>
<tr>
<td>C-Thiol</td>
<td>Thiol-GGAUCAAUCAUGGCAA</td>
</tr>
<tr>
<td>R-Thiol</td>
<td>Thiol-CAAAGUGCUUACAGAGC</td>
</tr>
<tr>
<td>TR-R-Thiol</td>
<td>Thiol-CAAAGUGCUUACAGAGC-TR</td>
</tr>
<tr>
<td>miR-17</td>
<td>CAAAGUGCUUACAGAGCAGGUAG</td>
</tr>
<tr>
<td>AntimiR-17</td>
<td>CUACCUCUCUGUAAGCACUUUG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>GAAGGTTAGGTTGTCGGAGTC</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GAAGATGGTGATGGGATTTTC</td>
</tr>
<tr>
<td>MAP2-F</td>
<td>GGGATTAGCATACACCAGCCG</td>
</tr>
<tr>
<td>MAP2-R</td>
<td>AGGCATCTGTCCTCAAAGTC</td>
</tr>
<tr>
<td>Nestin-F</td>
<td>GTGTAGAGGCCCCTGTTGG</td>
</tr>
<tr>
<td>Nestin-R</td>
<td>TCTTCTCCAGGGGTGACTC</td>
</tr>
<tr>
<td>Tuj1-F</td>
<td>TCCGGAGGTCTGTTCAAGCG</td>
</tr>
<tr>
<td>Tuj1-R</td>
<td>TCGGACACCAGGTCTTCC</td>
</tr>
</tbody>
</table>

All of the RNA strands were chemically modified by 2'F and provided by Bioneer® (Korea).

### 3. Results and discussion
3.1. Structure characterization of the Ag@Bi$_2$Se$_3$ core–shell NPs

For the nanoplatform, Bi$_2$Se$_3$ NP was synthesized and encapsulated with Ag (Ag@Bi$_2$Se$_3$). The morphologies of the synthesized Ag@Bi$_2$Se$_3$ core–shell NPs were investigated by TEM, as shown in Figure 2a and b. The high-resolution TEM image shows that well-dispersed, spherical, and uniform NPs without agglomeration formed. The average size of the as-synthesized Ag@Bi$_2$Se$_3$ core–shell NPs was about 50.5 nm.

The optical absorption properties of Bi$_2$Se$_3$ and Ag@Bi$_2$Se$_3$ in DMF/water at RT were investigated, and the results were compared with those of silver NP (AgNP, 50 nm). As illustrated in Figure 2c, Bi$_2$Se$_3$ NP showed optical absorption at 408 nm (band gap > 2.7 eV). Accordingly, Ag@Bi$_2$Se$_3$ displayed a red shift in its localized surface plasmon resonance band (~27 nm, in comparison with AgNP). The band had a broader range possibly because of the electron transfer between the Bi$_2$Se$_3$ NP and Ag shell.

The elemental composition of the as-synthesized Ag@Bi$_2$Se$_3$ NPs was also analyzed using X-ray energy-dispersive spectrometry (EDS). The EDS image (Figure 2d) indicates the coexistence of the elements Ag, Bi, and Se in the NP with a Bi/Se atomic ratio of 2:3. Furthermore, the X-ray powder diffraction (XRD) measurements were conducted to study the crystal structure and phase formation of the as-prepared Ag@Bi$_2$Se$_3$ NPs. As shown in Figure 2d (inset), the peaks in the XRD pattern illustrate the existence of the rhombohedra crystal phase of Bi$_2$Se$_3$, as reported earlier.$^{34}$ We observed two peaks close to 38 and 44, which are associated with [111] and [200] planes of silver (face-centered cubic; JCPDS card no. 04-0783); further proves the formation of Ag@Bi$_2$Se$_3$ NPs.

3.2. Analysis of plasmonic properties of Ag@Bi$_2$Se$_3$ NPs
The surface plasmon properties of the NPs were also investigated using SERS measurements. We used 4-MBA as the Raman reporter. Because of the size diversity of the NPs, the entire surface areas of the NPs were maintained equal. The NPs were treated with a compromised concentration of 4-MBA via silver–thiol conjugation. Afterward, SERS analysis was carried out on the prepared NPs. Figure 2e and the correlated statistical plots in Figure 2f demonstrate that the SERS signal of Ag@Bi$_2$Se$_3$ is about 2.3 times higher than that of AgNP (50 nm). Notably, the very low SERS signal of Bi$_2$Se$_3$ NP was mainly due to the weak efficiency of 4-MBA conjugation onto the NP. The acquired data confirm the function of the Ag shell in extracting the plasmonic properties of the Bi$_2$Se$_3$ NP. Though we have recently developed an 11 nm gold core–shell Bi$_2$Se$_3$ NP (Au@Bi$_2$Se$_3$) with almost the same properties, the size of the present Ag@Bi$_2$Se$_3$ is more appropriate for biomedical applications (30–200 nm); it accumulates more effectively inside the tumor tissues and has higher drug/gene loading capacity.

We observed optical properties of Ag@Bi$_2$Se$_3$ that are more stable compared with those of AgNP. We extracted 100 random aliquots from each NP solution prepared from one batch. The corresponding optical absorbance of the samples was recorded and plotted (Figure S1). As demonstrated, the relative standard deviation (RSD) of the data recorded for AgNP at 408 nm and Ag@Bi$_2$Se$_3$ at 435 nm were 9.6 % and 1.9 %, respectively. The higher signal stability of Ag@Bi$_2$Se$_3$ may be attributed to the role of Bi$_2$Se$_3$ as a TI to suppress the fluctuation and backscattering of the spin-polarized surface charges. These results which may need more comprehensive studies, demonstrate promising applications of Bi$_2$Se$_3$ for the development of reliable apparatuses for various research areas.
3.3. Structure formation analysis of 3WJ-RA/R

For the gene inhibition strategy, we utilized miRNA as a short non-coding RNA, which has been found to be involved in various cancers and disorders\textsuperscript{47,48}. Prior to the structure analysis of 3WJ-RA/R, we confirmed the formation of the RA/R structure by using a high-resolution mass spectroscopy technique. Considering the molecular weights of RA, RA/NHS, PDPH, RA/PDPH, and thiolated-R strand (300.43, 397.44, 229.32, 511.74, 5058.21 g·mol\textsuperscript{-1}, respectively), a clear peak at 5460.39 can be seen from the mass spectrum in Figure S2; it describes the successful 1:1 conjugation of the RA and R strand.

Polyacrylamide gel electrophoresis (PAGE) was used to confirm the structure of 3WJ-RA/R. The left panel in Figure 3a shows the formation of the 3WJ structure, while the right panel displays the definite constitution of the 3WJ-RA/R before and after the miR-17 invasion. It is evident in the last lane of the right panel that miR-17 had successfully displaced the RA/R strand, proving the performance of our gene inhibition mechanism.

The melting temperature (T\textsubscript{M}) analysis of the 3WJ-RA/R at different pH conditions was conducted to investigate the stability of the structure under various physiological environments. As shown in Figure S3a, T\textsubscript{M} values of 3WJ-RA/R were \textasciitilde61.2°C and \textasciitilde83.3°C, which were attributed to the dehybridization of the RA/R strand from the structure and the entire structural dissociation, respectively. In addition, at different pH conditions (4–9), both T\textsubscript{M} values remained almost at the same level, demonstrating that the 3WJ-RA/R can stay robust under different physiological environments (Figure S3b).

3.4. Structure analysis of Ag@Bi\textsubscript{2}Se\textsubscript{3}-3WJ-RA/R
Because of the high plasmonic activity of Ag@Bi₂Se₃, we used the SERS method to control the functionalization of the NP by its sequential conjugation with PVP and 3WJ-RA/R. Because of its amphiphilic characteristics, PVP could be physically adsorbed onto the NP surfaces via its hydrophobic chain to passivates the NP more effectively inside the PBS and cell medium. After the PVP treatment (500 nM), SERS measurements were conducted on the NP; the results are displayed in Figure 3b (black curve). We observed a distinct Raman peak at 463.6 cm⁻¹ describing the effective conjugation of PVP onto the NP surface. After that, the pre-hybridized 3WJ-RA/R was conjugated onto the PVP-modified Ag@Bi₂Se₃ through covalent Ag–S bonding to displace a certain amount of physisorbed PVPs. Because the weak Raman signal of the RNA compared with that of RA, we used the specific Raman peak of RA (1595.3 cm⁻¹) for the stoichiometry calculations. From the blue curve in Figure 3b, it is evident that after the conjugation of 3WJ-RA/R with Ag@Bi₂Se₃/PVP, another Raman band emerged at 463.6 cm⁻¹, which corresponded to the PVP. This illustrates the partial displacement of PVPs with 3WJ-RA/R. However, the Raman band at 1595.3 cm⁻¹ weakened in comparison with the red curve. The calculated optimum concentration for 3WJ-RA/R was 10.52 nM. According to the surface area of Ag@Bi₂Se₃ and the size of PVP10k and 3WJ-RA/R, the number of molecules attached to the Ag@Bi₂Se₃ was estimated to be ~272 and ~344 molecules (equivalent to ~1376 functional groups), respectively.

The size and zeta potential of the stepwise conjugation of Ag@Bi₂Se₃ with PVP and 3WJ-RA/R were also investigated; the results are shown in Figure 3c. As demonstrated, conjugation of Ag@Bi₂Se₃ with PVP and 3WJ-RA/R resulted in a gradual increase in the NP size from 50.3 nm to the average size of 59.7 nm. On the other hand, encapsulation of Ag@Bi₂Se₃ with PVP led to a positive shift in the zeta potential of the modified NP from −22.8 to −15.2 mV.
Whereas, the zeta potential of Ag@Bi$_2$Se$_3$/PVP shifted to a more negative value (−32.6 mV), after being conjugated with 3WJ-RA/R, which is within the appropriate stability range of the colloidal dispersions.

The optical absorption behavior of the modified NP was also monitored. The UV–Vis spectra of different modification steps are provided in Figure 3d. The UV–Vis measurements represented a slight reduction in the optical absorbance of Au@Bi$_2$Se$_3$/PVP, which was expected because of its more positive zeta potential compared with Ag@Bi$_2$Se$_3$. The final structure displayed a negligible change in its absorbance peak intensity, which suggests good dispersion properties of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R.

The colloidal stability of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R was tested using SERS and UV–Vis measurements on the NP in PBS (pH 7.4) for 3 weeks (Figure S4a and b). We observed an insignificant decrease in the optical absorption and Raman signal for the modified NP, suggesting its excellent colloidal stability. Moreover, the loading efficiency of RA on the modified NP was calculated to be around 1.4 μM for 120 μg·mL$^{-1}$ of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R (Figure S4c and d).

3.5. Endocytosis and endosomal/lysosomal escape ability of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R

In order to investigate the role of CPR on the cellular uptake (endocytosis) efficiency of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R, SH-SY5Y cells were treated with two kinds of NPs (Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R with and without the CPR moiety). The two NPs, the B-strands of which were 5'-labeled with Texas red (TR), enabled the in vitro fluorescence imaging of the NPs. The mean fluorescence intensity (MFI) of the uptaken NPs (see Figure 4a, Z-stack images) was calculated based on the region of interest analysis. The TR signal recorded for the
Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R without CPR was observed to be lower than that of NPs harboring CPR (Figure 4a and b). The endocytosis efficiency of the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R with CPR at a concentration of 120 μg·mL$^{-1}$ on 1 × 10$^6$ cells was calculated ~84 %, which was around 40 % greater than those prepared without CPR. Moreover, flow cytometry analysis was carried out to better understand the cellular uptake efficiency of the developed NPs. As seen in Figure 4c, Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R harboring CPR represented higher rates (81.4 ± 3.9 %) than NPs without CPR (47.2 ± 1.4 %) (Student t test, $p < 0.05$, $n = 3$). The acquired results, which are in consistent with the MFI study, clearly demonstrate the role of CPR in the cell internalization efficiency of the modified NPs (for experimental details, refer to the Supporting Information, section 1.a).

We also studied the cytotoxicity of the developed NPs on SH-SY5Y cancer cells (Figure S5). Compared with the bare Bi$_2$Se$_3$ NPs, Ag@Bi$_2$Se$_3$ dampened the cytotoxicity of the Bi$_2$Se$_3$ NP by 19.5 % for 48 hr at a concentration of 120 μg·mL$^{-1}$. Likewise, Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R displayed almost no toxicity after 48 hr of incubation. After 6 days of cell treatment with Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R, about 89 % of the cells were alive, demonstrating the high biocompatibility of the proposed structure. The acquired results are in good agreement with previous reports for the dose-dependent in vivo/in vitro toxicity of AgNPs and the role of PVP in the NP biocompatibility enhancement$^{50,51}$.

We tested the endosomal/lysosomal escape capability of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R. Interestingly, CPR could effectively disrupt the endosome/lysosome membrane after 7 hr of incubation. As seen in Figure S6, after 3 hr of incubation, a majority of the NPs were co-localized (white spots) with endosomes and lysosomes, demonstrating that almost all of the NPs were located inside the endosomes/lysosomes. After 7 hr of incubation, the co-localization was
reduced by approximately 88% because of the effect of CPR. Therefore, CPR could effectively facilitate the endocytosis/endoosomal escape of our developed NP.

The gene transfection efficiency of the Ag@Bi$_2$Se$_3$/PVP-3WJ was also investigated. The Lipofectamine®RNAiMAX reagent was used and loaded with antimiR-17. Ag@Bi$_2$Se$_3$/PVP-3WJ (without RA/R) was also conjugated with the same concentration of the 3WJ structure. Under identical conditions, SH-SY5Y cells were transfected with Ag@Bi$_2$Se$_3$/PVP-3WJ and Lipofectamine®RNAiMAX. After 6 days of incubation, the mRNA expression levels of the MAP2, nestin, and Tuj1 genes were measured and plotted in Figure S7. As it is evident, Ag@Bi$_2$Se$_3$/PVP-3WJ showed about 42% higher efficacy than the lipofectamine, illustrating the higher gene transfection efficiency of our developed NP.

3.6. Controlled-release monitoring of RA

Before the in vitro test was performed, the capability of the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R on gene inhibition in PBS containing miR-17 was monitored on the basis of the Raman spectrum recorded from the RA molecule. The strand displacement strategy was used for gene inhibition, from which the over-hanged toehold sequence of the B-strand assisted miR-17 to displace the RA/R strand and detach the RA molecule from the plasmonic Ag@Bi$_2$Se$_3$ NP, leading to a considerable drop in the RA Raman signal. Figure 5a depicts a different time-point SERS spectrum recorded from Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R in PBS (pH 7.4) containing miR-17. After 3 hr of NP treatment, the intensity of the Raman peak at 1595.3 cm$^{-1}$ (RA) declined by ~88%, while the Raman peak at 463.6 cm$^{-1}$ (PVP) stayed almost constant, indicating the selective displacement of the RA/R strand by miR-17 without affecting the PVP stoichiometry.
In order to prove our hypothesis of pre-requisite drug delivery mechanisms, we designed another A-strand possessing a scrambled toehold sequence. Under identical conditions, SH-SY5Y cells were treated with both types of NPs of normal (Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R) and scrambled toehold sequence (Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R (Scr)). Figure 5b–d illustrates the recorded SERS spectra recorded from two types of NPs. As seen in the case of normal NPs (Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R), the intensity of the Raman peak at 1595.3 cm$^{-1}$ (RA) dropped by ~92 % after 48 hr of incubation, whereas the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R (Scr) harboring a scrambled toehold sequence showed about 8 % deduction in the SERS signal after 48 hr followed by a gradual decrease to 31 % after 6 days of incubation. Accordingly, the obtained data confirm our hypothesis for the prerequisite RA delivery mechanism.

We studied the dissociation of RA from the R strand, which was based on the disulfide breakage strategy. RA/R was dissolved in PBS (pH 7.4) and media containing GSH. After incubation and filtration, the purified strands were collected, and their optical absorbance at 360 nm (characteristic absorbance peak of RA) was measured. Five identical sets of experiments were performed to test the disulfide breakage efficiency as a function of GSH incubation time. As depicted in Figure S8a, after 2 and 5 hr of incubation, the optical absorbance of the samples prepared in PBS and media respectively dropped to ~98.2 % and ~96.3 % of their initial values. We also performed similar experiments on Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R. After incubation of the NPs with PBS (pH 7.4) containing GSH ± miR-17, samples were purified and subjected to UV–Vis measurement. It should be noted that after the designated incubation time, samples were first centrifuge-purified then subjected to filtration. Afterward, a flow-through solution was added to the centrifuged NPs and directed to the optical absorbance measurement. As seen in Figure S8b, samples treated with GSH and miR-17 showed disulfide breakage after 7 hr of incubation.
Notably, the longer RA dissociation process (compared with Figure S8a) was due to the step for the miR-17 inhibition. On the other hand, the samples incubated with GSH only did not show a significant decrease in the optical absorbance of RA, further proving our pre-requisite drug delivery mechanism.

We studied RA dissociation from the R strand in vitro. RA/R was labeled with TR fluorescent dye at its 3’ end. In order to avoid overlapping fluorescent signals, the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R having the unlabeled B strand was assembled. The intrinsic blue fluorescent feature of RA (excitation wavelength: 405 nm) enabled its visualization inside the cells. After 3 hr of NP incubation (Figure S9), the RA and R strands were mostly co-localized inside the cytoplasm (white color); whereas in 24 hr, some of the RAs (yellow color) were observed to be separated from the R strand (magenta color), tending to accumulate inside the nucleus. Moreover, the morphological change of the cells after 24 hr can be clearly seen in the bright-field images, which represent the onset of cell differentiation. It should be noted that, it would be difficult to clearly visualize the spatial–temporal point process of the RA delivery because of the dynamic involvement of RA in further biological pathways and the degradation probability of the R strand inside the cytoplasm. Nevertheless, the obtained data confirm the dissociation of RA from the R strand inside the cytoplasm (for experimental details, refer to the Supporting Information, section 1.b).

3.7. Analysis of cell differentiation efficiency of the NP-treated SH-SY5Y

In the final experimental step, we investigated the effect of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R on the neural differentiation efficiency of SH-SY5Y cells. The SERS measurement was utilized to monitor the neural differentiation of SH-SY5Y cells in a nondestructive manner. As depicted in
Figure 5e, after 2 days of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R incubation, Raman bands of the RA molecule were totally demolished. However, after 6 days (full differentiation), new Raman bands appeared at the region between 750 and 1550 cm$^{-1}$ besides the band for the PVP molecule. The change in the Raman spectrum represented an alteration in the concentration and existence of biochemical components such as proteins, nucleic acids, amino acids, and lipids$^{31,52}$. The peaks at 960, 1001, 1097, 1214, 1284, 1422, and 1498 cm$^{-1}$ were assigned to proteins (C–C str.), amino acid (Phe), lipid (chain C–C str. and PO$^{2-}$ str. of nucleic acids), amide III β-sheet Trp/Phy str., amide III/C–O str. of carbohydrates, as well as DNA/RNA bases of G and A. Although Raman spectroscopy has been utilized to analyze many types of cells, it is still uncertain whether different cell types and fates could be profiled on the basis of their distinguished Raman spectra.

The expression level changes of the neuronal markers MAP2, nestin, and Tuj1 genes were also measured for 9 days (Figure 5f). After 6 days of NP incubation, the expression of neuronal markers reached a maximum level and remained almost steady up to 9 days. The results indicate that 120 µg·mL$^{-1}$ of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R could successfully up-regulate the expression level of all three neural markers, further supporting our previously obtained data.

We characterized the neural differentiation via MAP2 immunolabeling. As seen in Figure 5g, no neurite outgrowth after 6 days of incubation was observed for the PBS control and Ag@Bi$_2$Se$_3$/PVP-treated cells. However, free RA could poorly differentiate the cells because of the weak cell penetration efficiency of RA. Likewise, Ag@Bi$_2$Se$_3$/PVP-RA/R resulted in partial differentiation and promoted cell apoptosis (reducing the cell population) due to the fast release of a high dosage of RA inside the cells. Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R (Scr) effected 12% of neural differentiation, whereas the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R could fully differentiate the SH-SY5Y cells into neurons after 6 days of incubation without requiring tedious steps of media.
exchange, which could cause possible cell loss and contamination. These results also support our proposed drug delivery mechanism for the pre-requisite release of the RA and efficient neural differentiation of SH-SY5Y cells.

The role of antimiR-17 onto the selective differentiation induction of SH-SY5Y cells was also investigated. The negative control, Ne4C cells, were incubated with Ag@Bi$_2$Se$_3$/PVP-3WJ (without RA/R) for 6 days, and the results were compared with identical data recorded from SH-SY5Y treated with the same NPs. As evident in Figure S10, the SH-SY5Y cells after 6 days of NP incubation started to grow axons, but no neuritic growth was visible in case of the Ne4C cells. These results further confirm the role of antimiR-17 in the selective differentiation induction of SH-SY5Y cells.

Interestingly, we could observe cell-to-cell differentiation, in which the differentiated cells containing NP were attempting to find and differentiate the undifferentiated cells by growing their axons toward them. Two typical examples of cell-to-cell differentiation after 36 hr of NP transfection are shown in Figure 6a and b. The cells containing NP were already differentiated and about to differentiate the undifferentiated cells located in their vicinity. As seen in the 3D image of Figure 6b, the NPs are clearly localized inside the differentiated cells, whereas the undifferentiated ones contain no NPs. Although the observed phenomena would interest various researchers in different fields of neural networking, it requires more comprehensive research to further understand the mechanisms and applications.

4. Conclusion

In summary, the novel Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R nanobiohybrid material was used to differentiate SH-SY5Y cancer cells into neurons after 6 days with the ability of real-time drug
release and cell fate monitoring in a nondestructive fashion. The fabricated NP consisted of a SERS-active Ag@Bi$_2$Se$_3$ core–shell NP as the imaging agent and a newly developed three-way junction RNA structure (3WJ) as the quad-functional agent. The 3WJ was composed of three legs to address the stoichiometry challenges of multi-functionalization of NPs in a highly controllable nature. One leg of the 3WJ was conjugated with Ag@Bi$_2$Se$_3$, the second one harbored a CPR and a fluorescent tag, and the third one had a therapeutic role. The mechanism of the therapeutic leg was based on a new paradigm for releasing RA slowly in a time-dependent and pre-requisite manner, in which the RA release requires the miR-17 inhibition step. The slow release of RA along with the miR-17 inhibition enabled a high throughput of neural differentiation with almost no apoptosis induction. The developed NP with a controllable multi-functionalization ability and the proposed drug delivery mechanism can be easily tuned for any type of cancer for fabricating robust cancer differentiation agents in the near future. The observed phenomena for the cell-to-cell differentiation would definitely interest various researchers to study and develop many devices and apparatuses in the field of bioelectronic medicine.
Figure 1. Schematic diagram of the Ag@Bi₂Se₃/PVP-3WJ-RA/R fabrication and its application in NB cell differentiation. R stands for the small RNA strand conjugated with RA.
**Figure 2.** a) and b) TEM graphs of the as-synthesized Ag@Bi$_2$Se$_3$, c) UV–Vis spectra of the three indicated NPs, d) EDS spectrum and XRD pattern (inset) of Ag@Bi$_2$Se$_3$, e) SERS spectra of 4-MBA conjugated with Bi$_2$Se$_3$, AgNP, and Ag@Bi$_2$Se$_3$. f) Plot of the corresponding Raman intensity recorded for 4-MBA on the studied NPs; spectra obtained using 785 nm laser at 1 sec exposure time and data were analyzed on the basis of average signals of 20 points from three independent samples.
Figure 3. a) Native PAGE (12 % TBM) with ethidium bromide staining for 3WJ-RA/R conformation analysis. “M” stands for the 100-base-pair DNA marker. b) Typical SERS spectra of the step-by-step modification of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R. c) Plot of the size and zeta potential of different modified NPs; data were obtained from three independent measurements. d) UV–Vis spectra of the different modified NPs.
Figure 4. a) CLSM analysis of the endocytosis efficacy of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R with and without CPR after incubation with SH-SY5Y cells for 2 hr. Detection was done by using immunofluorescent images comprising actin (2- Alexa flour™ 546 Phalloidin, green) and nucleus (1-Hoechst, blue). The RNA (B strand) was fluorescence-tagged with Texas red (TR) at its 5ʹ terminal; co-localization of the cytoplasm and fluorescent NPs can be clearly observed in the magnified z-axis images. b) Plot of the corresponding MFI of the TR extracted from the two types of NPs uptaken by cells; * $p < 0.05$ (n = 3) vs. control. c) Flow cytometry analysis of SH-SY5Y cells treated with Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R with and without CPR for 2 hr normalized to PBS control. The RA/R strand was labeled with TR at its 3ʹ end, whereas the B strand was unlabeled. Cell population percentage was evaluated on the basis of $p < 0.05$ (n = 3) vs. control.
**Figure 5.** Typical Raman spectra of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R before (0 min) and after incubation with a) PBS (pH 7.4) containing miR-17 and b) SH-SY4Y cells at different time points. c) Raman spectra for Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R harboring scrambled toehold sequence.
before and after incubation with SH-SY5Y cells. d) Plot of the normalized Raman intensity of RA at 1595.3 cm$^{-1}$ as a function of incubation time of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R with PBS and SH-SY5Y (normal and scrambled). e) Typical SERS spectra recorded from the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R for durations of 0, 2, and 6 days of incubation with cells. f) Plot of the relative abundance of three neuronal marker proteins (MAP2, nestin, and Tuj1) as a function of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R incubation time. g) Neural differentiation of SH-SY5Y treated with different modified NPs for 6 days. The cells were immunostained with MAP2 (green) for the neurons. Statistical data was obtained based on three independent experiments.
Figure 6. Two typical examples (a and b) of cell-to-cell differentiation after 36 hr of Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R transfection. The yellow arrows show the differentiation directions; inset 2 illustrates the 3D image of the cell-to-cell interaction along with a high magnitude image to display the NP locations inside the differentiated cells.
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Author Contributions

The manuscript was written through contributions of all authors. M. M., T. L., and J.-W.C. designed the project. J. Y. and H. K. C. synthesized and characterized the cell works and in vitro works. V.P. and B. B.G. synthesized the NPs.

Notes

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Supporting Information

Flow cytometry experiment, analysis of RA dissociation from R strand, optical properties of AgNP and Ag@Bi$_2$Se$_3$, mass spectrum of RA-PDPH-RNA conjugation, melting temperature analysis of 3WJ-RA/R, SERS spectra and optical properties of the Ag@Bi$_2$Se$_3$/PVP-3WJ-RA/R, cell viability assay, visualization of the endosomal/lysosomal escape, expression level analysis of neuronal markers and control experiment on Ne4C cells.

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