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Bismuth-Based Nanomaterials: Recent Advances in Tumor Targeting and Synergistic Cancer Therapy Techniques

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# Abstract

Despite all of the efforts in the field of cancer therapy, the heterogeneous properties of tumor cells induce an insufficient therapeutic outcome when treated with conventional monotherapies, necessitating a shift in cancer treatment from monotherapy to combination therapy for complete cancer treatment. Multifunctional bismuth (Bi)-based nanomaterials (NMs) with therapeutic functions hold great promise for the fields of cancer diagnosis and therapy based on their low toxicity, X-ray sensitive capabilities, high atomic number, near-infrared driven semiconductor properties, and low cost. Herein, a comprehensive review of recent advances in various medicinal aspects of Bi-based NMs is presented including: evaluation of in-tumor site accumulation, tumor targeting, and therapeutic performance, as well as the characteristics, benefits, and shortcomings of Bi-based NM-mediated major monotherapies. In addition, the cooperative enhancement mechanisms between two or more of these monotherapies are described in detail to address common challenges in cancer therapy, such as multidrug resistance, hypoxia, and metastasis. Finally, this review opens new insights into the design of multimodal synergistic therapies for potential future clinical applications of Bi-based NMs.

# 1 Introduction

Cancer is a common term denoting malignant neoplasms in which cells abnormally divide and grow without control. These cells are able to metastasize into other tissues, and, thus, cancer is currently one of the leading causes of death worldwide. In 2012, according to universal estimates, cancer accounted for about 8.2 million deaths. At present, cancer has become a great concern in the world as the mortality rate is anticipated to increase to 12 million by 2030. Despite all of the efforts and expenditures that have been made to develop new anticancer therapies, tumoral tissues properties (such as complexity, variety, and heterogeneity) largely limit the efficiency and effectiveness of current clinical treatments.[1] Specialists in the fields of material science, biology, chemistry, physics, pharmacy, biology, and medicine have formed multiple groups to develop new ways to detect and treat early-stage cancerous tissue.

Nowadays, major clinical approaches for cancer therapy include chemotherapy (CTX),[2] radiotherapy (RT),[3] and high intensity focused ultrasound (HIFU) therapy,[4] which produce promising results by suppressing tumor cell proliferation extending patient survival. Also, previous studies have proved the effectiveness of photodynamic therapy (PDT)[5] in treating nonsmall cell lung cancer and esophageal cancer. In addition, preliminary clinical studies have demonstrated the high anticancer efficacy of approaches such as photothermal therapy (PTT),[6] immunotherapy,[7] gene therapy (GT),[8] and magnetic hyperthermia (MHT).[9] Although all are still in preclinical stages, these approaches have created a great deal of hope for the clinical treatment of cancer in the future.

Results obtained from both exploratory studies and clinical practices have revealed that a single method is unable to wholly eradicate the tumor or efficiently prevent tumor metastases due to the heterogeneity of tumor tissue, which consists of cancerous cell subpopulations resistant to monotherapy and internal cancer stem cells we still have to determine how to kill.[10] An alternative method is integrating two or more types of therapies as a combined therapy, which has been introduced to overcome the weaknesses of monotherapy. Synergistic treatments rely greatly on the incorporation of monotherapeutic benefits within one nanosystem instead of simply combining methods to amplify their cooperative effects. Swift advances in the field of nanotechnology have permitted the assembly of several types of therapeutic probes into a single nanostructure.

Prominently, nanoscale materials show numerous noticeable advantages (**Figure** **1**). First, nanomaterials (NMs) can accumulate and favorably stay in the tumor site at a high concentration for an extended period of time through the enhanced permeability and retention (EPR) effect.[11] Second, the surface of nanoparticles (NPs) can be modified with diverse functional groups—such as peptides, proteins, and other biomolecules—to decrease nonspecific uptake via the reticuloendothelial system (RES) and, in contrast, to enhance accumulation via the receptors overexpressed in tumor cells.[12] Third, nanomaterials with large surface-to-volume ratios can be used to carry high payloads of drugs, genes, and other therapeutic agents and biomolecules and protect their degradation in complex physiological environments.[13] Also, therapeutic agents can be released from the functionalized nanomaterials under a controlled manner using either internal or external stimuli (e.g., pH, GSH, light, etc.), which largely prevents the early outflow of therapeutic agents within nontarget tissues, reducing unwanted side effects.[14] Further, the large surface area of nanoparticles permits an enhanced reaction for photothermal therapy and diagnostic tools such as magnetic resonance imaging. For this purpose, several types of nanomaterials have been developed to co-load two or more types of therapeutic agents for multimodal synergistic therapy, which significantly enhance therapeutic efficacy and effectively increase therapeutic sensitivity in malignant tumors with resistance to monotherapeutic approaches.

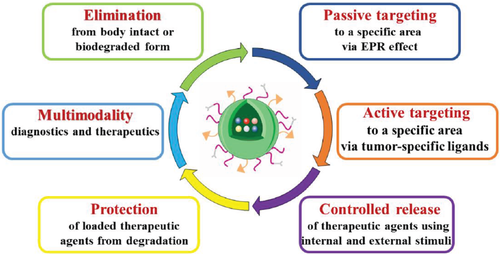
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Figure 1 Advantages of nanoprobes in medicine.

Due to the increasing clinical importance of multiple synergistic therapies, understanding the underlying synergistic mechanisms of different combination therapeutic approaches is extremely helpful to establish more effective tumor treatments. Among the developed NPs, bismuth (Bi; as the heaviest stable and nonradioactive element on the periodic table) has attracted considerable attention for cancer treatment, due to its acceptable toxicity, being less expensive, and having high X-ray attenuation capability and NIR absorption coefficients. For the first time, this review focuses on the tumor accumulation of Bi-based NMs and their underlying mechanisms for cancer therapy, in order to design therapeutic agents based on Bi NPs for more effective cancer therapy for the future.

Based on the increasing biomedical applications of Bi-based NMs, and also the lack of a comprehensive source of detailed novel findings in the field, we tried here to present a broad review of the recent advances of Bi-based NMs in various medical aspects. Major topics and gaps in knowledge are highlighted in this paper as compared to previous surveys and are presented as follows: at first, the evaluation of the tumor accumulation of Bi-based NMs in in vitro and in vivo studies is explored. Subsequently, to evaluate the therapeutic performance of Bi-based NMs accumulated in the tumor site, the characteristics, benefits, and shortcomings of Bi-based NM-mediated major monotherapies are introduced. In addition, the cooperative enhanced mechanisms between two or more of these monotherapies are described in detail to mitigate tumor-to-therapy resistant properties, such as multidrug resistance (MDR), hypoxia, and metastasis (**Figure** **2**). Finally, this review presents comprehensive views into the elaborate design of novel therapeutic agents based on Bi NPs with high treatment effectiveness by co-integrating a tumor accumulation/synergetic therapeutic effect within a single nanoprobe for a Bi NM-mediated multimodal synergistic therapy for improved clinical applications of the future.

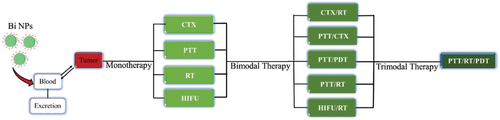
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Figure 2 Summary of this review.

# 2 Passive Tumor Targeting via the EPR Effect

Taking into account that tumoral tissues have unique structures (such as a leaky vascular structure, hyper vasculature, and impaired lymphatic drainage), nanoprobes are able to selectively home into a primary tumor region and even its metastatic region at high concentrations, and for prolonged retention times compared to molecular probes, without any extra targeting ligands, due namely to the EPR effect.[15] The EPR effect is considered as the main reason nanoprobes accumulate in tumors for greater biomedical imaging and therapy of cancerous tissues.[15] For the first time, in 1986, Matsumura and Maeda explained the selective distribution of protein–polymer conjugates into tumoral tissues by the EPR effect.[16] To date, micelles, plasma proteins, DNA-complexes, liposomes, and even nanoprobes have demonstrated a tumor homing capability.

Studies on tumor accumulation efficiency of organic macromolecules have indicated size-dependent EPR effects. In other words, biodistribution profiles show that only macromolecules with a size larger than 5 nm, which are nonrenal clearable, have a passive targeting effect in tumoral tissue.[17] The EPR effect has been found to be negligible in the case of molecules excreted via the urinary system, inducing an inefficient tumor homing ability and fast tumor clearance. As a first reason, the concentration of renally excreted molecular probes sharply reduces in the blood during a short time, inducing lower accumulation probes within tumors. As a result, both the low biodistribution within pretumoral organs and renal clearance efficiency act as the major factors to increase tumor accumulating efficiency, following long-term blood circulation. The next reason is their very short retention time inside tumoral tissues, due to their small form which can easily go from the tumor into the bloodstream.[18]

The conjugation of active homing ligands (such as peptides and proteins) on molecular and nanoprobe surfaces effectively increases their targeting efficiency and retention half-life in tumors. Common nanoprobes are not able to pass renal filtration, while polyethylene glycol (PEG) polymers exhibit resistance to confiscation by the RES organs; thereby, PEGylated nanoprobes have a longer blood circulation half-time and higher concentration compared to those of molecular probes, resulting in a higher tumor targeting capability via the EPR properties of tumors.

As an example, PEG-Bi nanospheres capped with thiol ligands (Bi-SR)[19] and PEGylated copper bismuth sulfide nanorods (PEG-Cu3BiS3)[20] (NRs) selectively accumulated in 4T1 tumors with maximum passive homing efficiencies of 4.6 and 7.1%ID g−1 at 24 h p.i., respectively. A targeting efficiency of 10% after 24 h was measured for PEGylated iron diselenide-decorated bismuth selenide (PEG-FeSe2/Bi2Se3 NPs),[8] and only 4.58 µg PEG-modified polypyrrole (PPy)-coated Bi nanohybrids (Bi/PPy NPs)[21] per gram tumor accumulated in the 4T1 tumor. For the C6 and HeLa tumor model, the accumulating efficiencies of PEG-gadolinium-chelate functionalized Bi[21, 22] and PEG-Bi nanocrystals[23] reached 8 and 8.9%ID g−1 at 1 and 24 h p.i., respectively. Cu3BiS3 NPs[24] and Bi subcarbonate nanotubes/Bi subcarbonate nanoclusters (BNTs/BNCs)[25] with a polyvinylpyrrolidone (PVP) coating showed a negligible EPR effect with a value of 0.75 and 4.0%ID g−1 in HeLa and Huh-7 tumors, respectively, but a Bi2Se3 spherical-sponge[26] with the same coating showed a higher uptake by HeLa tumor, as high as 9.66%ID g−1 after 12 h p.i. and 15.0%ID g−1 Bi nanodrugs (NDs)[27] in U14 tumors after 24 h p.i . For the 4T1 tumor model, the values of non-PEGylated Bi NPs, such as bovine serum albumin (BSA)-Bi2S3 NPs[28] and BSA-Bi2Se3 NDs,[29] were determined to be 8.3 and 5.0%ID g−1 during 24 h p.i., respectively, while this value was much lower for tween-Bi2S3 NRs[30] (0.9%ID g−1, 24 h p.i.), indicating a weak EPR effect. The tumor uptake of nonrenal clearable Bi-based nanoprobes was found to be 0.75–10%ID g−1 at 1–24 h p.i. It is worth noting that PEG polymers serve as a cellular-interaction-resistant coating, thereby, the cells in general are not able to efficiently internalize PEGylated NPs.

## 2.1 Active Tumor Targeting via Ligands

As a strategy, active accumulating ligands are widely used to conjugate with PEGylated NPs. The active targeting efficiency of Bi NPs, like other NPs, is significantly higher than passive targeting efficiency. The conjugation of a cyclic nine amino acid peptide, CGNKRTRGC (LyP-1), with PEG-Bi2S3 NPs[31] and PEG-Bi NPs[32] in 4T1 tumor enhances their homing efficiencies from 3.2 and 3.66%ID g−1 to 8.4 and 6.26%ID g−1, respectively, corresponding to a 2.6 and 1.7 times increase (**Figure 3a**,**b**). The PEG coating only enhanced tumor uptake of Bi NPs compared to non-PEGylated Bi NPs by 1.4-fold. Active targeting leads to a robust enhancement of tumor targeting efficacy in a SKBR-3 tumor model when the Trastuzumab (Tam) antibody is used as a targeting ligand on bismuth sulfide@mesoporous silica (Bi2S3@mPS) core–shell NPs.[33] The capability of taking up nontargeted NPs (PEGylated NPs) was found to be only 12.44 µg g−1 after 24 h, but surprisingly, the ultrahigh tumor uptake of 195.2 µg g−1 was found in Tam-Bi2S3@mPS NPs at the same time, corresponding to a 16 times targeting efficiency (Figure **3c**). Tam is known as a human epidermal growth factor receptor 2 (HER-2) monoclonal antibody, which specifically identifies the HER-2 receptor on HER-2 positive SKBR-3 breast cancer cells, resulting in significant tumor uptake.

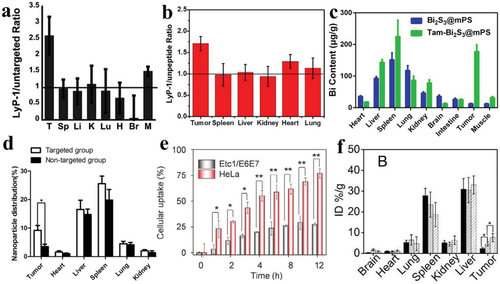
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Figure 3 Biodistribution of a) LyP-1-PEG-Bi2S3 NPs. Reproduced with permission.[31] Copyright 2011, Wiley-VCH. b) LyP-1-PEG-Bi NPs. Reproduced with permission.[32] Copyright 2017, American Chemical Society. c) Tam-Bi2S3@mPS NPs and d) folate-perfluorohexane NPs carrying Bi2Se3 in the main organs of tumor-bearing mice. c) Reproduced with permission.[33] Copyright 2018, Wiley-VCH. d) Reproduced with permission.[34] Copyright 2016, The Royal Society of Chemistry. e) The uptake efficiency of RGD-CS-Bi2Se3 NSs by HeLa and Etc1/E6E7 cells after different time periods. \*\*p < 0.01 or \*p < 0.05. Reproduced with permission.[35] Copyright 2017, Nature Publishing Group. f) Biodistribution of Bi NPs (black), RBC Bi NPs (dense), and F-RBC Bi NPs (sparse) in the main organs of tumor-bearing mice. Reproduced with permission.[36] Copyright 2018, Elsevier.

When perfluorohexane (PFH) nanoparticles carrying bismuth sulfide[34] were linked with a folate active ligand, the HeLa tumor could confiscate 9%ID g−1 during 3 h, which was ≈2.25 times higher than that of the nonfolate-targeted group (≈4%ID g−1) (Figure **3d**). Within 12 h, the efficient accumulation of Bi2Se3-chitosan (CS) NPs[35] targeted with an arginylglycylaspartic acid (RGD) active peptide in HeLa tumor cells was higher than those of Etc1/E6E7 normal cells, with a magnitude of 2.8 (Figure **3e**). The longer circulation of red blood cell membrane (RBC)-modified Bi NPs[36] persuaded a richer tumor uptake through passive targeting, as confirmed by the higher tumor uptake of RBC-Bi NPs (4.53%ID g−1) compared to Bi NPs (2.29%ID g−1). In contrast, the conjugation of a tumor-specific targeting folate ligand could further improve the accumulation of Bi NPs within 4T1-tumors (7.59%ID g−1), inducing a 1.7 and 3.3 times increase over RBC-Bi NPs and Bi NPs, respectively (Figure **3f**). The tumor targeting efficiency of Bi NPs with various active ligands after 24 h postinjection is listed in **Table** **1**.

**Table 1.**List of Bi NPs conjugated with active accumulating ligands for enhanced tumor homing 24 h postinjection

|  |  |  |  |
| --- | --- | --- | --- |
| **Type of NP** | **Ligand** | **Tumor** | **Tumor targeting efficiency [%ID g−1]** |
| Cu3BiS3 NDs[61] | PEG | 4T1 | 7.1 |
| Bi nanospheres[19] | SR-PEG | 4T1 | 4.6 |
| FeSe2/Bi2Se3 NPs[8] | PEG | 4T1 | 10.0 |
| Bi/PPy NPs[21] | PEG | 4T1 | 4.58 µg g−1 |
| Gd Bi NPs[22] | PEG | C6 | 8.0 |
| Bi nanocrystals[23] | PEG | HeLa | 8.9 |
| Cu3BiS3 NPs[24] | PVP | HeLa | 0.75 |
| Bi2Se3 spherical-sponge[26] | PVP | HeLa | 9.66 at 12 h |
| Bi NDs[27] | PVP | U14 | 15.0 |
| BNTs/BNCs[25] | PVP | Huh-7 | 4.0 |
| Bi2S3 NPs[28] | BSA | 4T1 | 8.3 |
| Bi2Se3 NDs[29] | BSA | 4T1 | 5.0 |
| Bi2S3 NRs[30] | Tween | 4T1 | 0.9 |
| Bi2S3 NPs[31] | PEG-LyP-1 | 4T1 | 8.4 |
| Bi NPs[32] | PEG-LyP-1 | 4T1 | 6.3 |
| Bi2S3@mPS[33] | Tam | SKBR-3 | 195.2 µg g−1 |
| PFH-Bi2S3[34] | Folate | HeLa | 9.0 at 3 h |
| Bi2Se3-CS NPs[35] | RGD | HeLa | 4.0 |
| Bi NPs[36] | Folate-RBC | 4T1 | 7.6 |

According to the results, the ratio of the tumor to the liver of nonrenal clearable Bi-based NMs was low, due to their higher accumulation in RES organs (e.g., liver and spleen). There was a tumor-to-liver ratio lower than 0.55 for the nonrenal clearable Bi nanoprobes, with the average value at about 0.3.

As a result, after conjugation of the Bi-based NMs with active targeting ligands, a significant difference was observed for their accumulation in the liver and spleen, as a tumor-to-liver ratio of 0.59 (passive targeting: ≈0.3; active targeting: ≈0.59). Although renal-clearable and biodegradable Bi NPs are favorable for biomedical applications, their tumor targeting efficiency is another important issue that should be considered. For the biodegradable PVP-Bi2Se3 NPs, the passive targeting efficiency of Bi ions in a U14 tumor model reached almost a steady value after 24 h with a maximum value of 7.4%ID g−1 (1.48 µg g−1) after 72 h, like other nonrenal clearable Bi NPs, while a value of 0.42 µg g−1 was found for the Se ions. The biodegradable BNTs,[25] with a renal clearance efficiency like renal-clearable NPs, and original nanoclusters of BNC were accumulated in Huh-7 tumors with a targeting efficiency of 8.09 and 5.88%ID g−1 after 1 h. As time went on, the concentration of BNC gradually decreased to 4.5%ID g−1 after 9 h, unexpectedly, and an ultrahigh uptake of BNTs by tumor cells was observed with a targeting efficiency of 18.5%ID g−1. For both the BNTs and BNCs after 24 h, only 4%ID g−1 was measurable. The biodegradable selenocysteine-modified Bi2Se3 NPs (PVP-Bi2Se3@Sec NPs) not only greatly accumulated in BEL-7402 tumors, with a tumor uptake ≥60 nmol for Se and Bi elements, but also the tumor-to-liver ratio was >1, indicating strong tumor-selective distribution. Although the liver is a major site for the accumulation of Se, the tumor-to-liver ratio was found to be 1.8 and 1.2 after 24 and 72 h postinjection, respectively. The tumor-to-liver ratios of Bi element sharply increased to reach ratios higher than 6, due to the high accumulation of Bi in the kidneys. After 7 days, the Se and Bi elements still showed a ratio of 0.45 and 2.4, respectively.

# 3 Classification and Characteristics of Major Cancer Monotherapies

Perhaps the treatment of cancer can be considered as the most successful application of NPs of all biomedical applications. The benefits of NPs are from its low side effects, strong tumor targeting capability and bioavailability, and overcoming common drawbacks seen in conventional therapy techniques. The eight types of therapies including CTX, PDT, PTT, RT, HIFU, GT, and magnetic hyperthermia are the main types of monotherapies, in which nanotechnology has significantly helped. To date, CTX, RT, and HIFU have been able to achieve clinical success by suppressing the growth of the tumor and prolonging the survival of the patient. Also, PDT can effectively help to treat lung and esophageal cancer. Although other monotherapies are still not used clinically, numerous laboratory and preclinical studies have revealed their great anticancer properties with promising outcomes for future clinical translation. To date, various forms of Bi-based NMs (such as nanospheres, NDs, and nanosheets (NSs)) have been widely utilized for the first five monotherapies. In this section, the role of Bi as an agent therapeutic for the corresponding monotherapies is described in detail.

## 3.1 Chemotherapy

The destruction of cancerous cells using drugs is called CTX. Docetaxel (Dtxl), cisplatin (CDDP), doxorubicin (DOX), and paclitaxel (PTX) have been extensively used as CTX drugs, as tumor cells are rapidly killed in their presence. They are nonspecifically distributed to the whole body and then rapidly cleared through the urinary system, reducing the effectiveness of chemotherapeutics and systemic toxicity. Often after the long-term use of chemotherapeutics, cancerous cells display MDR. In order to solve these problems, drugs have been incorporated into carriers through absorption, conjugation, adsorption, and encapsulation processes to reduce their concentration needed for killing cancer cells (and, thus, minimize MDR). These drug delivery systems (DDSs) provide a promising strategy for the accumulation of drugs into the tumor site using non/active targeting mechanisms. The EPR effect facilitates drug delivery to the tumor by DDSs due to the porous vasculature as well as inadequate lymphatic drainage of the tumor, which consequently causes the uptake of DDSs efficiently into the tumor microenvironment than normal tissues resulting in more retention. The size of the vasculature pores is different for all types of cancer, determining the EPR effect.

For the next mechanism, active targeting ligands can effectively contribute to further improve the tumor-specific homing efficiency of DDSs.

The first application of nanomaterials in the cancer therapy field was reported as drug carriers to improve delivery and therapeutic efficacy. Immunosuppressants, steroids, anesthetics, antibiotics, painkillers, vaccines, and CTX agents can be carried by NPs as portable drugs. To date, different types of DDSs based on organic and inorganic materials have been developed to deliver the drug to tumoral tissue. Owing to acceptable biocompatibility and biodegradability, some organic DDSs (such as liposomes, dendrimers, micelles, and polymers) have been introduced into clinical trials, but poor stability and immunogenicity restrict their future application. Although desirable properties of inorganic NP-based DDSs (known as inorganic DDSs) like high drug loading potential and delivery of drugs, biostability and low inducing immune-responses have converted them into better DDSs, they exhibit low circulation time, high cytotoxicity, and fast clearance by RES. Therefore, organic/inorganic hybrids can integrate beneficial properties of both DDSs types for effective drug delivery to a tumor.

To date, DDSs based on Bi NMs only have been loaded with DOX.[25, 26, 33, 37] Graphene and its derivatives, such as silica with a mesoporous structure, have been used as an excellent candidate to increase drug loading capacity of the Bi-based DDSs with the following benefits: large surface area, high biocompatibility and π–π stacking interaction with DOX.[34, 37] Recently, Bi-based nanovehicles with mesoporous structure have been developed.[25, 26, 37] The inherent porous nanostructure not only allowed for high drug loading but also prevented the existence of an extra mesoporous coating on the NP surface. For effective DOX loading, in addition to the hollow structure of inorganic nanomaterials, polymer coatings with a negative charge are suitable due to the positive charge of DOX.[37] Generally, the loading capacity of Bi2Se3 spherical-sponge NPs (19.1%),[26] PEGylated Bi2Se3 nanourchins[37] (37.9%), and PEGylated hollow Cu3BiS3 NPs[37] (60.8%) were comparable to rGO/Bi2S3 NPs[37] (≈500%), silica/Bi NPs[37] (40–50%), and Bi2S3@mPS[37] with large pore sizes (as 60.85 mg/100 mg DOX) .

The delivery of antitumor drugs loaded into DDSs to the target tissue is the most important issue in CTX. As a unique advantage, the nano-DDSs are able to choose the drug release site, called “controlled release.” The stimulus responsive drug vehicles react to either internal (such as pH and redox level) or external (such as ultrasound waves, temperature, and electromagnetic waves caused by an external source) agents and specifically release the drug into the tumor site. All the developed Bi-based nanovehicles showed an unstable mode inside the tumoral tissue. The tumor microenvironment contains a pH lower than normal tissue yet, the amino protonation and solubility of DOX molecules increases in an acidic environment. Finally, the hydrophobic interaction between the Bi-based nanovehicles and DOX molecules decrease, causing tumor-specific drug diffusion. Furthermore, the mesoporous network can accelerate the process of drug release from the Bi-based nanocarriers. A burst drug release from the Bi-based nanocarriers was found under an external energy source, such as NIR irradiation. When the PEGylated Bi2Se3@SiO2[37] was triggered by NIR irradiation, DOX release efficiency reached 32% during 1 h, which was 128% higher than that of the non-NIR-triggered group. The exposure of rGO/Bi2S3 NPs[37] to the NIR irradiation enhanced the incorporated DOX release from 23.2% to 72.53%, corresponding to a 313% increase. Generally, in drug therapy, it is crucial to pay attention to two steps, the first being the distribution of drug carriers in the body, which preferably accumulates inside a tumor site. The second is activation of the release of the trapped drug from the carrier into the surrounding environment by simulation, in which external simulation is a better choice to achieve localized drug release. In the case of an external stimulus, it should be noted that their operating time is proportional with the maximum intracellular accumulation of the drug for effective therapy. Therefore, the nano-DDSs not only play an effective role in drug delivery and its release control to the target tissue, but they also reduce the MDR of malignant cells. Moreover, an integration of a cocktail of drugs into DDSs can successfully ablate the resistance of tumoral tissue, where a single drug may fail. Although many studies have been carried out on inorganic nanodrug vehicles in recent years, none have been successfully used for clinical applications.

## 3.2 HIFU Therapy

The energy of noninvasive ultrasound (US) waves can be deposited locally to a target tissue which provides a sound-thermal treatment paradigm. HIFU therapy can induce the irreversible damage of cells and destroy vessels of the tumor like a bloodless surgical knife. Although a negligible effect has been found for a single US beam, the focus of several US beams on a point can produce significant thermal and mechanical effects by converting sound-to-thermal effects. Thanks to the easy penetration of US beams into soft tissue, HIFU may offer an effective treatment for all deep-located tumors with any depth limitation. Additionally, real-time US imaging allows for the monitoring of tumors and the guidance of the treatment process at time points before, during, and after HIFU therapy, providing a real-time response of treatment effectiveness. HIFU mainly works based on two mechanisms: heat generation and acoustic cavitation. The target tissue absorbs US radiation and converts it into heat to ablate the focused volume of the tumor through heating of the cancer cells.

The magnitude of the deposited US energy directly determines the generated heat amount. A rapid temperature increase within a tumor occurs following the deposition of US beams with high-power, which can provide adequate heat to thermally eradicate the tumor. Concurrently, focused US beam generate microbubbles that grow and finally implode, known as the acoustic cavitation phenomenon. Since the temperature inside the microbubbles is very high, the collapse of all of the microbubbles that appeared is accompanied by shockwave induced irreversible mechanical cell damage. Hence, both thermal and mechanical effects cooperatively improve the treatment efficacy of HIFU when exposed to powerful US beams.

Despite the high-performance of HIFU to induce tumor cell necrosis, the US waves rapidly attenuate with increasing penetration depth, which limit the employment of HIFU for ablation of deep tumors. To solve this problem, HIFU enhancement agents (EAs) have been strongly suggested to increase deep treatment efficiency. Commercial organic microbubbles used for US imaging can concurrently be used as HIFU EAs. The improved thermal and acoustic effects are found after generation of echogenic gas bubbles. However, the large micrometer size of the microbubbles in inducing short blood retention times and insufficient tumor homing, decrease in vivo applications. Recently, Bi2S3-embedded lipid NPs encapsulated thermosensitive perfluorohexane (BS-PFH-NPs)[34] have been used for improved HIFU therapy (**Figure** **4a**). The phase transition from liquid to gas due to the elevation of temperature occurred once the NPs were exposed to the HIFU. Although the phase transformation temperature of PFH is 29 °C, an improved vaporization temperature was observed for PFH after lipid encapsulation. When the increase in temperature continued to 73 °C, all the microbubbles began to implode and eventually disintegrated, inducing the cavitation-mediated mechanical effect.

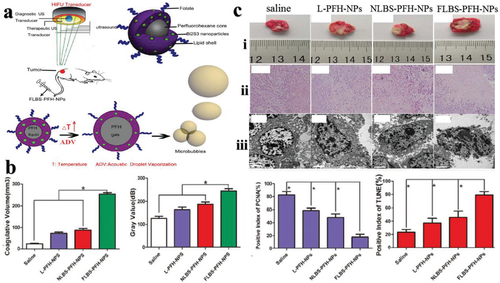
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Figure 4 a) Schematic of the fabrication of perfluorohexane NPs carrying Bi2Se3 for HIFU therapy. b) Quantitative analysis of HIFU therapeutic efficacies on the degassed bovine livers after the injection of different agents. c) HIFU therapeutic efficacies on HeLa tumor-bearing mice after the administration of different agents: i) TTC staining (necrotic tissues: gray; nonablated tumors: red), ii) H&E staining, and iii) TEM images of the tumor tissues collected from the mice at the end of HIFU therapy and after injection of different agents. Scale bar is 2 µm. \*p < 0.05. Reproduced with permission.[34] Copyright 2016, The Royal Society of Chemistry.

For degassed bovine livers in vitro (Figure **4b**), the gray level, coagulative volume, apoptosis index of proliferating cell nuclear antigen (PCNA) and TUNEL-representing apoptosis demonstrated the great potential of Bi2S3 NPs to make the cavitation more effective and change the acoustic impedance, with higher efficiency observed for the folate-targeted group inducing an enhanced absorption of US dependence on Bi2S3 NPs. Therefore, integration of Bi2S3 NPs and PFH synergistically promoted the HIFU therapeutic effect. By necrosis specific triphenyltetrazolium chloride (TTC) staining, a significant large necrotic volume of tumor tissue (white) was found after co-treatment with NP/HIFU (Figure **4c**-**i**). Most importantly, the necrotic region was sharply distinguished form nondamaged tissues (red), which was attributed to the localized tumor destroying the capability of nanoagents without damaging the adjacent healthy tissues. Through microscopic examination of the malignant tissues (Figure **4c**-ii), no remarkable coagulative necrosis was observed for the samples treated with saline while for the other three groups, the necrotic regions were distinguished from non-necrotic tissue by observable lines. Lysed cell membranes and fragmented nuclei were observed as typical cell damage signs after coagulated necrotic tissue. The irreversible cellular damage in the targeted regions upon co-treatment with NPs and HIFU was reconfirmed by transmission electron microscopy (TEM) (Figure **4c**-iii). Meanwhile, some of the saline-received cells showed an intact structure with condensed chromatin.

Despite protein denaturation and coagulation via necrosis which resulted from hyperthermia serving as a major killing effect for HIFU therapy, HIFU can concurrently disrupt and change the function and structure of cell membranes by the generation of cavities, and finally ablate malignancy by corresponding shock waves and jet flow. Furthermore, the mechanical effects (acoustic streaming and shear stress) as well as other HIFU ablation enhancement factors are strongly dependent on the number of generated microbubbles. More importantly, Bi2S3 NPs can not only operate as a potent cavitation source, it can reduce the cavitation threshold to improve the cavitation effect of HIFU. Therefore, for effective HIFU ablation, simultaneously utilizing Bi2S3 and HIFU yields can be an appropriate candidate.

## 3.3 Radiation Therapy

RT, as one of the most common therapy techniques, is able to effectively suppress the growth—or even destroy malignant cells in many tumors—using ionizing radiation. According to the type of radiation source, the RT mainly is classified into two forms of internal radioactive therapy (IRT) and external beam therapy (EBT). In IRT, both initial and metastatic tumors are exposed to innate radiation from radioactives (such as 131I, 188Re, 177Lu, 90Y, etc.) injected into the circulatory system, in contrast, in EBT, high energy rays (including protons, electrons, and photons) with a generation source outside of the body are employed.

Here, we especially focus on X-ray therapy (XRT), which is generated by photon sources. After irradiation, the energetic X-ray photons interact with the exposed tissue and indirectly ionize their atoms by the photoelectric phenomenon, as the X-rays carry no charge. Because the possibility of the photoelectric phenomenon is directly related to the fourth square of an atomic number, the Bi heavy metal can deliver a considerably higher fraction of the deposited energy per mass, causing the target to be exposed to a localized dose. Although the photoelectric effect is the dominant phenomenon for XRT with energy in the kilovoltage (kV) range, kV radiation travels a short distance inside the body, causing an excessive deposited dose to the skin. Therefore, megavoltage (MV) radiations are usually used for the treatment of deeper tumoral tissues. Given that a small fraction of photons induce the photoelectric effect upon MV radiation, Bi NMs mediated radiosensitizing is expected with a negligible enhancement ratio, as calculated by Monte Carlo.[38] The survival curve of cells treated by Bi NPs upon 10 MV radiation showed a dose enhancement ratio of 1.25.[38] This difference can be justified by processes occurring within the biological tissue followed by ionizing radiation. The chemical, physical, and biological processes are introduced as RT efficiency enhancement pathways (**Figure** **5a**),[39] whereas in the theoretical predictions by Monte Carlo, only the physical phase are involved in the Bi NPS + XRT-induced sensitivity enhancement ratio (SER).[38] Therefore, Bi-based NMs are becoming potential radiation sensitizers due to their high X-ray absorption ability (**Table** **2**).

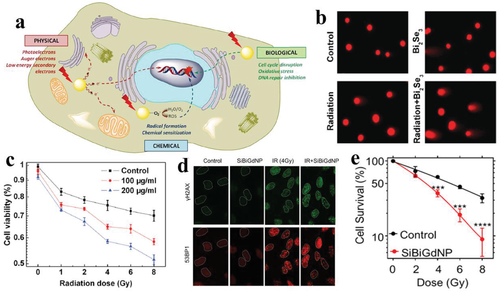
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Figure 5 a) Mechanisms of heavy metal-based nanoprobes radiosensitization. Reproduced with permission.[39] Copyright 2017, Elsevier. b) Fluorescent images of DNA of HeLa cells. c) The cytotoxicity of different treatments on HeLa cells. Reproduced with permission.[40] Copyright 2014, Wiley-VCH. d) Qualitative analysis of γ-H2AX and 53BP1 foci formation after different treatments. e) Colony formation of A549 cells after different treatments. \*\*\*\*p < 0.0001 or \*\*\*p < 0.001, \*\*p < 0.01, and \*p < 0.05. Reproduced with permission.[41] Copyright 2017, American Chemical Society.

**Table 2.**List of Bi compound-based nanomaterials for enhanced RT

|  |  |  |  |
| --- | --- | --- | --- |
| **Types** | **External** | **Internal** | **Representatives** |
| Pure Bi NPs[36, 41] | + | − | Si-BiGd, Bi |
| Bi2Se3 NPs[35, 40, 42] | + | − | Bi2Se3 nanoplates, Bi2Se3 sheet, Bi2Se3 sphere |
| Bi2S3 NPs[62] | − | + | PLGA/Bi2S3 |

For example, the DNA damage to the cells radiosensitized with Bi2Se3 nanoplates[40] was clear in acquired fluorescent images (Figure **5b**). The DNA stain has a long tail in the RT + Bi2Se3 group, indicating remarkable DNA damage, while there was no such visible tail for those treated with/without Bi2Se3 NPs or even those exposed to only ionizing radiation, also cell viability followed a radiation dose and concentration-dependent trend (Figure **5c**).[40] The double strand breaks (DSBs) are the most lethal DNA lesion among different types of DNA damage, which lead to clonogenic cell killing. Since the formation of a phospho-Ser139 (γ-H2AX) antibody is one of the primitive steps in response to DSBs, the γ-H2AX foci assay is used for sensitive detection of DNA damage (Figure **5d**).[41] The analysis of experimental DNA DSBs provides evidence regarding the fact that DNA repair inhibition could be a promising mechanism to increase the sensitivity and efficiency of Bi NPs + RT (Figure **5e**).[41]

Moreover, monitoring of caspase activation indicated the involvement of both receptor (extrinsic) and mitochondrial (intrinsic) death pathways in Bi NPs/RT-induced apoptosis (**Figure** **6a**).[35] However, the higher activity of caspase-9 (Figure **6b**) and Mito-tracker red (Figure **6c**) highlighted the major role that mitochondria play in the Bi NP-induced radiosensitization effect. The co-treatment effect of Bi NPs and X-rays on intracellular apoptotic amplification was confirmed by an increase in the phosphorylation level of proteins—such as histone, proapoptotic kinase p38 and p53, which are all involved in the DNA damage-mediated p53 apoptosis—whereas the antiapoptotic protein kinases, including protein kinase B (AKT) and extracellular-signal-regulated kinase (ERK), have been suppressed (Figure **6d**). Furthermore, the expression levels of vascular endothelial growth factor receptor 2 (VEGFR2) and methylguanine-DNA methyltransferase (MGMT), as DNA repair proteins, decreased, which led to the inhibition of cellular self-repair. Also, Bi NPs + XRT significantly decreased the expression of thioredoxin reductases (TrxR), leading to an imbalanced intracellular redox and increased efficacy of RT sensitization (Figure **6e**). TrxR has an important role in the regulation of apoptosis, and redox balance and TrxR inhibition can lead to DNA damage in a reactive oxygen species (ROS)-dependent manner. In conclusion, Bi-based NMs attenuate the cellular repair pathways of p53, MAPKs and AKT as well as enhance X-ray damage to the cells as mentioned above (Figure **6f**).

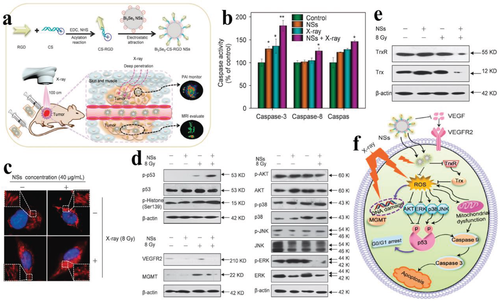
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Figure 6 a) Schematic of Bi2Se3-CS-RGD NSs synthesis for cancer radiation therapy. b) Caspase activation graph of HeLa cells for the control, NSs, X-ray, and X-ray + NSs groups. c) The changes in mitochondria morphology and d) the expression levels of apoptotic signaling pathways of treated cells. e) Protein expression levels of TrxR and Trx in the cells. f) Proposed apoptotic signaling pathways after treatment with radiation and Bi2Se3-CS-RGD NSs. \*\*p < 0.01 or \*p < 0.05. Reproduced with permission.[35] Copyright 2017, Nature Publishing Group.

Besides a radiosensitizing capability of Bi-based NMs, Bi2Se3 NPs fill a potential role as a scavenger of radiation-induced free radicals or antioxidants. Due to their superb electrocatalytic activity, Bi2Se3 NPs[42] effectively cleared the generated ROS and protected C57BL/6 mice to high-energy gamma rays (**Figure** **7a**). When mice were exposed to a fetal dose of 7.5 Gy, all of the experimental mice died after 14 days; surprisingly, their survival fraction amplified to 71% after co-treatment with Bi2Se3 NPs. To understand the radiation protection, the investigation of the underlying mechanisms is helpful. The superoxide dismutase (SOD) enzyme has three isoforms in mammals with different localization. This enzyme is an effective antioxidant defense system against superoxide anion (O2−), which alternately promotes conversion of O2− to hydrogen peroxide (H2O2) or to the O2 molecule. Since 3,4-methylenedioxyamphetamine (MDA) is considered a negative byproduct, SOD and MDA could be used as indicators for the response levels of ionizing radiation. The ROS clearing capability of Bi2Se3 NPs induced recovery of SOD (Figure **7b**) and MDA (Figure **7c**) lung and liver tissue in comparison to the irradiated mice without Bi2Se3 NPs.

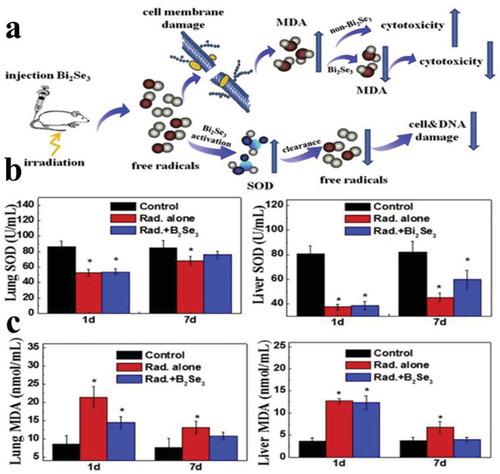
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Figure 7 a) Mechanism of the radioprotector effect of Bi2Se3 NPs. b) SOD and c) MDA graph during 7 days after different treatments. \*p < 0.05 (compared to controls at the same time period). Reproduced with permission.[42] Copyright 2017, Elsevier.

## 3.4 Photothermal Therapy

Hyperthermia is known to increase temperatures to a range of 41–47 °C for tens of minutes after application, which is considered a fatal temperature range for cells. Tumoral tissues show a heat tolerance lower than normal tissue due to poor blood supply, therefore, hyperthermia acts as an effective destroyer for tumoral tissues at this temperature range. Hyperthermia-induced cell damage is irreversible, as it weakens cell membranes by denaturing proteins. Hyperthermia using light laser has been a revolution in the therapy field of cancerous tissues, enabling thermal damage in the target region under a controlled and limited manner. In PTT, as a hyperthermia-based phototherapy method, the tumor tissue exposed to a laser absorbs the light energy laser and converts it to localized heat for the ablation of malignant cells. Infrared (IR) lasers are the best choice for the therapy of deep tumors, due to the tissue composition of water, lipids and hemoglobin having the lowest absorption in the range of 700–1300 nm (known as the IR region), leading to deeper penetration levels up to several centimeters. However, nonspecific heating of PTT is a major issue limiting its future application for clinical purposes, which not only destroys tumor tissue but also healthy tissue.

Nanoexogenous agents with a strong NIR harvesting capability as a photothermal agent (PTA), can more efficiently convert the deposited photo into thermal energy. The plasmonic property of Bi is called a nanoconfinement property due to the appearance of this property at the nanosize. A higher photothermal efficiency is found for NPs with a direct bandgap; bismuth chalcogenides (Bi2E3, E = S and Se) benefit from this property. The bandgap of Bi2Se3 NPs is 1.3 eV, providing a superior efficiency than conventional PTAs, such as Au and Pd NPs. To date, a number of Bi-based PTAs have been employed for single PTT (**Table** **3**), which are mainly Bi, Bi2Se3, Bi2S3, and Cu3BiS3-based NMs with photothermal efficiencies of about 28–35%.

**Table 3.**List of NIR-absorbing PTAs based on Bi nanocompounds

|  |  |
| --- | --- |
| **Types** | **Representatives** |
| Bi2S3 NPs[20, 44, 63] | Bi2S3 nanoflowers, Bi2S3 nanospheres, Bi2S3 NDs, Bi2S3–Au NRs |
| Bi2Se3 NPs[30, 43, 64] | Bi2Se3 NRs, Bi2Se3 nanoplates, GO/Bi2Se3 nanosheets, Bi2Se3 nanosheets |
| Cu3BiS3 NPs[24, 65] | Cu3BiS3 nanospheres, Cu3BiS3 NDs |
| Bi NPs[21-23] | Bi NDs, Bi nanocrystals, PPy/Bi nanospheres, Gd-Bi nanospheres |
| Nanocomposite[66] | Urchin-shaped Bi2S3/Cu2S/Cu3BiS3 NPs |

The synergistic effect of Bi-based NPMs and laser radiation could significantly persuade tumor cells to undergo a series of damaging processes like cell shrinkage, loss of contact (**Figure** **8a**), and lastly a significant loss in tumor weight (Figure **8b**). Despite the high thermal killing effect of Bi-based NMs, when conjugated with the polymer PPY,[21] the photothermal conversion efficiency (η) of pure Bi NPs shifted form 30.4% to a high value of 46.3%. The localized surface plasmon resonance (LSPR) property of common semiconductors with a narrow bandgap is mainly attributed to their large number of free carriers (around 1021–1023 cm−3). However, the free carrier density of Bi2S3 NPs was found to be low, with a value of 1015–1016 cm−3. In other words, the oscillation frequency of Bi2S3 NMs, inducing a localized surface plasmon resonance effect, placed at the microwave or terahertz region. Therefore, the oscillation frequency of NIR light cannot trigger the photothermal property of Bi2S3 NPs. These intrinsic deep level defects (DLDs) are known as a key factor in the photothermal mechanism of Bi2S3 NPs.[43] For nano-semiconductors, DLDs, particularly in those with a low number of free carriers, potentially recombine nonradiatively electron–holes after exposure to produce phonon emission and thermal effects, as stated by the Shockley–Read–Hall recombination theory. In the case of Bi2S3 NPs, the generation of phonons and heat takes place via both sulfur vacancy (VS) and the BiS antisite (Bi replacing S) pathway of DLDs (Figure **8c**-A).[43] Interestingly, the photothermal property of Bi2S3–Au NRs, apart from the original DLDs, was mainly related to the BiS defects formed as mediated by Au S replacement with Bi atoms. Concurrently, the Au Fermi level significantly helps to trap more electrons into BiS defects, in which both pathways amplified the number of generated phonons, resulting in higher photo-to-thermal efficiency (Figure **8c**-B).

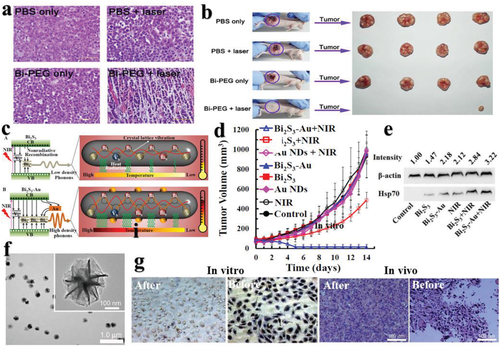
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Figure 8 a) Images of tumor sections and b) size of tumors of mice for each group. Reproduced with permission.[23] Copyright 2017, Elsevier. c) Graphical illustration of how A) Bi2S3 and B) Bi2S3–Au NRs under NIR irradiation have the photothermal effect. d) Average volume of tumors as a function of time. e) HSP70 expression level after different treatments. Reproduced with permission.[43] Copyright 2018, Wiley-VCH. f) The images of crumpled-paper-like Bi2S3 nanoflowers for PTT. g) Images of Bi2S3 nanoflower-treated HeLa cells and tumor slices before and after NIR laser irradiation. Reproduced with permission.[44] Copyright 2016, Springer Nature.

The heat-induced lethality of the Au NDs on the 4T1 tumor tissue was negligible, whereas a strong inhibition potency of tumor growth was observed for both groups receiving Bi2S3 NPs, and further for Bi2S3–Au NRs, confirming the more potent PTT capability of Bi2S3–Au NRs (Figure **8d**). To evaluate the generated heat within the exposed cells at the molecular scale, the synthesized level of heat-responsive shock protein 70 (HSP70) was examined (Figure **8e**). A weak HSP70 expression was found after treatment with the NIR laser alone; in sharp contrast, co-treatment of laser irradiation with both Bi2S3 and Bi2S3–Au NRs induced a significant increase in the expression level of HSP70, where the Bi2S3–Au NRs acted as a stronger agent than Bi2S3 NRs. Hyperthermia can induce apoptosis by the suppression of the NF*κβ* signaling pathway through nitric oxide (NO) or by the continuous triggering of caspases related to tumor necrosis factors.

Recently, Bi2S3 nanobelts[44] with a bandgap of 1.22 eV was introduced. By assembling the crumpled-paper-like NSs to form 3D Bi2S3 nanoflowers, the bandgap value decreased to 0.8 eV (Figure **8f**). This decrease can be attributed to a mirror behavior of the nanoflowers, which significantly improved the fraction of reflection and absorption of the light laser. It is surprising that the corresponding photothermal conversion efficiency was shifted from 36.5% to the highest reported value of 64.3%. For a temperature increment of 34 °C, a concentration of 100 ppm Bi2S3 nanoflowers was equivalent with 760 ppm Bi2S3 nanobelts for a power density of 1.0 W cm−2, causing a 6.6-fold decrement. The Bi2S3 nanoflowers cooperatively resulted in cellular necrosis, including karyopyknosis, cytoclasis, and coagulation for the improvement of the effect of photothermal therapy in vitro and in vivo (Figure **8g**), whereas there were no cellular morphology changes for the untreated group.

# 4 Nanotechnology for Bimodal Synergistic Therapy

Both clinical and experimental results have shown that a single therapeutic technique is insufficient to completely eliminate a tumor, as well as it is unsuccessful to inhibit metastasis of cancerous cells. The heterogeneous properties of tumoral tissue acts as a dominant reason for the monotherapy resistance effect, due to the subpopulations of cancerous cells forming a tumoral tissue with a distinct morphological and phenotypic profile. As a model, the tumor cells exhibit resistance to drugs after long use, following unsuccessful CTX. The lack of oxygen inside the target tissue reduces the sensitivity of the tissue to ionizing radiation, leading to low RT efficiency. The advantages and disadvantages of the above cancer monotherapy are summarized in **Figure** **9**.

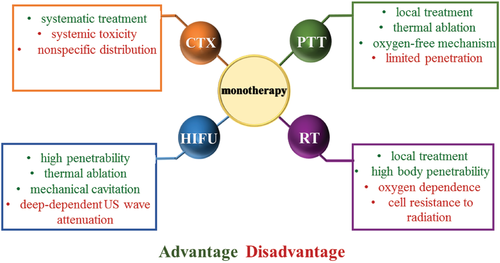
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Figure 9 Various modalities and their properties for cancer monotherapies reported for Bi NPs.

According to this, the combination of the advantages of two monotherapies may overcome the disadvantages of each monotherapy, inspiring the co-delivery of two types of monotherapy to encourage the benefits and offset shortcomings. Combination therapy is the integration of the synergistic benefits of monotherapies inside the tumor site to wholly destroy it. Therefore, shifting the cancer treatment from a monotherapy to a combination therapy has gained increased research attention to achieve complete cancer treatment. Today, the combined therapies have been possible with the advent of multifunctional NPs assembling therapeutic properties within a single system. After this point, an explosion of synergistic therapy studies using a wide variety of multifunctional nanoprobes has been reported. In this section, Bi NM-mediated synergistic therapeutic mechanisms will be introduced and discussed in detail (**Figure** **10**).

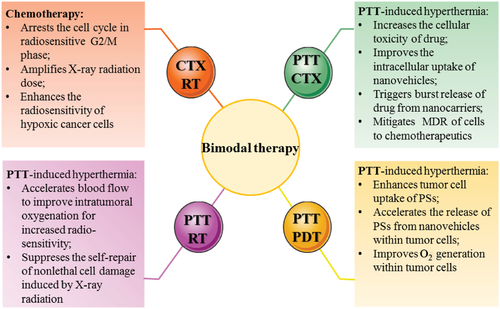
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Figure 10 Diagram of the proposed action mechanism for bimodal synergistic therapy for Bi NPs.

## 4.1 CTX-Based Bimodal Synergistic Therapy

In the case of Bi-based NMs, amplification of CTX efficiency has been achieved by co-treatment with RT, which is called synergistic chemo-radiotherapy (CTX/RT). In this subsection, the synergistic mechanisms behind CTX-RT and related studies will be described in detail.

### **4.1.1 CTX-Enhanced RT**

The co-treatment of anticancer drugs with ionizing radiation have demonstrated a synergistic effect for clinical purposes, known as CTX-RT. Currently, CTX-based RT is used as an effective strategy for the eradication of a wide variety of malignant tumors. Concurrent CTX tends to increase the sensitivity of tumor cells to radiation through interference in the mechanisms behind RT—namely, preventing the repair of radiation-induced sublethal cell damages and arresting the cells in the G2/M phase of the cell cycle, which have the most sensitivity to radiation. The synergistic effect not only controls the tumor area and its metastasis, but it also increases the survival of patients compared with radiation therapy alone.[25, 37]

Apart from the high atomic number of Bi to improve the ionizing radiation therapy effect by generating a greater number of secondary electrons, which increases DNA damage upon exposure to ionizing radiation, it is able to increase the generation of ROS inside cells to amplify ionizing radiation-mediated cell apoptosis. On the other side, DOX is introduced to promote the generation of intracellular ROS and interfere in the activity of DNA topoisomerase II. The ROS generation potency of Bi and DOX was confirmed via a fluorescent probe,[25] dichlorodihydrofluorescein diacetate (**Figure** **11a**), in which the intensity of fluorescence is considered as an ROS indicator in the cell cytoplasm and nucleus. Visible ROS content was found in the BNTs, X-ray irradiation, BNTs + RT, and BNTs + DOX groups, whereas co-treatment of DOX-loaded BNTs and X-radiation showed superb fluorescence intensity, offering a synergistic therapeutic potential.

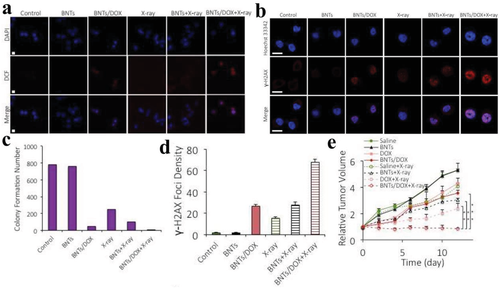
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Figure 11 a) CLSM images of Huh-7 cells to visualize the level of oxidative stress after different treatments. b) CLSM images of γ-H2AX foci formation in the nucleus of the cell for various groups. Scale bar is 20 µm. c) Colongenic assay of cells for all treatment groups. d) The number of γ-H2AX foci for each group. e) Average tumor volume of mice versus time. Reproduced with permission.[25] Copyright 2018, American Chemical Society.

The fluorescent intensity of Hoechst 33342 and H2AX foci formation, as a biomarker of cell nucleus fragmentation and DNA double-strand breaks, respectively, showed cellular damage induced by X-rays and DOX (Figure **11b**). X-ray mediated CTX efficiency was clear due to an increase in the fluorescent intensity of γ-H2AX foci. After co-treatment of DOX and X-rays, the intensity of γ-H2AX foci significantly improved, though it was lower compared with the BNTs/DOX + X-ray group, representing the synergistic effect, as well as more cellular uptake mediated by BNTs. The synergistic interactions between BNTs/DOX and X-rays almost completely suppressed the colony formation of Huh-7 cells (Figure **11c**). Based on the quantified DSB damage potency for each group (Figure **11d**), the BNTs/DOX + X-rays could generate a significant number of γ-H2AX foci, as high as 2.45- and 4.41-fold than those exposed by the BNTs/DOX and X-ray only, respectively. In in vivo evaluations (Figure **11e**), the synergistic effect of drug delivery and the tumor targeting of BNTs could suppress tumor proliferation of mice treated by the BNTs/DOX in comparison with those treated by the free DOX. There was a further suppression upon exposure to X-rays, and the highest inhibition was observed for the BNTs + X-ray group. A decrease of 83.5% in tumor weight was found for the drug containing BNTs in combination with the X-ray radiation group, indicating a more effective treatment compared to those in the DOX-loaded BNTs (32.2%) and BNTs without drug in combination with the X-ray radiation (42.4%) groups.

Although, in general, tumor cells are more sensitive than those in the majority of the rest of the body due to their rapid cell division, this is not always true. The oxygen content in tumoral tissue is low, known as hypoxic tissues, and therefore less sensitive to X-rays because most of the lethal damage is mediated by the free radicals produced by ionizing oxygen. Furthermore, the low oxygen level in cells may be the main cause of uncontrolled tumor growth in some cancers. Therefore, apart from the synergistic effect of CTX-RT, the integration of DOX and Bi within a single system as an exogenous agent for CTX-RT is a better choice, due to their potency to generate more ROS inside tumoral cells, which effectively improves the co-treatment of DOX and Bi efficiency.

## 4.2 PTT-Based Bimodal Synergistic Therapy

In this section, studies based on Bi NMs will be introduced to show that during hyperthermia, Bi NMs not only ablate tumor cells in an invasive manner, but they also improve the synergistic outcome of merged modalities like PTT/CTX, PTT/RT, and PTT/PDT.

### **4.2.1 PTT-Enhanced CTX**

Hyperthermia alone effectively kills tumor cells at >45 °C, whereas co-treatment of PTT with other therapeutic techniques induce such cell damage at 39–42 °C. The improvement of anticancer drug therapeutic effects by hyperthermia is introduced as thermo-chemosensitization. The synergistic effect of chemothermal therapy has been achieved through several pathways. Hyperthermia is able to increase tumor accumulating efficiency as well as the toxicity potential of drugs. The increased intracellular uptake of drugs and more drug-sensitivity of cells are underlying mechanisms for the enhancement of cellular toxicity. P-glycoprotein (P-gp) expression and multidrug resistance proteins can decline, which successfully improve the sensitivity to the drug in MDR-exhibited cells. P-gp is a vital transmembrane protein to enhance the penetrability of the cell membrane, causing the transportation of many substances out of the cell. Although photothermal therapy is sufficient to completely eliminate prime tumors, it is incapable to work in the case of metastatic tumor cells. In contrast, common CTX systematically kills both prime tumor cells and their metastases. Hence, co-treatment of CTX and PTT can not only ablate local tumors mediated by thermal effects but can also effectively suppress metastatic tumors even located at distant sites. Cooperative thermal- and chemotherapy based on Bi NMs and their synergistic mechanisms to enhance therapeutic effect are documented below (**Table** **4**).

**Table 4.**List of Bi compound-based nanovehicles for enhanced PTT/CTX efficiency

|  |  |  |
| --- | --- | --- |
| **Types** | **Drug** | **Representatives** |
| Silica-coated Bi NPs[33, 37] | DOX | rGO/Bi2S3, SiO2/Bi2S3, Si/Bi2S3 |
| Mesoporous Bi NPs[25, 26, 37] | DOX | Bi2Se3 nanospherical-sponge, Bi2S3 nanourchin |
| Polymer-Bi NPs[37] | DOX | PEG-Cu3BiS3, polydopamine/Bi2Se3 |

A synergistic CTX/PTT therapy effect was evident by making cooperative use of Bi2Se3 NPs and DOX (**Figure** **12a**).[45] After NIR irradiation of polydopamine (PDA)/human serum albumin (HSA)/DOX-coated Bi2Se3 NPs (Bi2Se3@PDA/DOX/HAS NPs)-treated HeLa cells, both the cytoplasm and nuclei of cells revealed a higher fluorescence intensity of DOX compared to a nonexposed group (Figure **12b**). The Bi2Se3 NP-mediated hyperthermia effect allowed the activation of a fraction of the internalized NPs via increasing the cell membrane permeability and cellular metabolism, causing protein denaturation and disruption of lysosomal membranes that release lysosomal enzymes into the cytoplasm, which also prompted the further release of DOX into the cell. Correspondingly, considerable karyopyknosis—representing apoptotic cell death with clear nucleus-related abnormal fragments—was observed for HeLa cells treated by either Bi2Se3@PDA/DOX/HSA NPs or free DOX (pointed by the yellow arrows), where the DOX in Bi2Se3@PDA/HSA NPs triggered more apoptosis death than free DOX (Figure **12c**). As observed by the infrared thermal images (Figure **12d**), the red color (representative of the highest temperature) began to appear at the tumor site mediated by Bi2Se3 NPs, and the temperature reached 47–49 °C within 10 min, which was sufficient to cause a hyperthermia effect and also promote drug release.

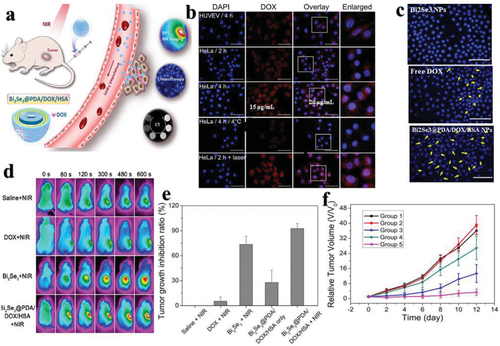
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Figure 12 a) Schematic of how Bi2Se3@PDA/DOX/HSA NPs can affect cancer cells. b) Uptake of DOX into HUVEC and HeLa cells for various treatment times. c) Morphology changes (yellow arrows) in HeLa cells caused by various treatment. Scale bar is 200 µm. d) In vivo thermal imaging of mice treated by different agents under NIR laser radiation. e) The ratio of tumor growth suppression under different treatments. f) Images of mice and the changes in corresponding tumor volume; Groups 1, 2, 3, 4, and 5: saline + NIR, free DOX + NIR, Bi2Se3 + NIR, Bi2Se3@PDA/DOX/HSA only, and Bi2Se3@PDA/DOX/HSA + NIR, respectively. Reproduced with permission.[45] Copyright 2015, American Chemical Society.

The synergistic chemothermal effect of Bi2Se3@PDA/DOX/HSA NPs showed an ability to suppress in vivo tumor growth at a ratio of 92.6% (Figure **12e**). The toxicity effect mediated by Bi2Se3 NPs + laser and Bi2Se3@PDA/DOX/HSA NPs without a laser was only 73.6% and 24.8%, respectively. The tumor's growth for those in the Bi2Se3@PDA/DOX/HSA NPs + laser group were successfully inhibited at a low *V*/*V*0 rate of 3.4, while with time, the growth of tumors for the Bi2Se3 NPs + laser irradiation (*V*/*V*0 of 13.3) and only laser irradiation (*V*/*V*0 of 26.8) groups were fast. The highest *V*/*V*0 value of 36.1 was for the control group. The tumor growth rate decreased during the Bi2Se3 NPs + NIR monotherapy period but was insufficient to ablate complete tumors without recurrence. Interestingly, using the therapeutic advantages of Bi2Se3@PDA/DOX/HSA NPs in combination with NIR radiation effectively reduced the tumor volume, even if any residual tumors were found after the course of treatment, indicating a successful co-treatment CTX/PTT (Figure **12f**). The Bi nanovehicles showed great drug delivery and photo-to-thermal harvesting capability, which may serve as an effective nanoagent to clinically ablate cancer cells in the future.

### **4.2.2 Photothermal-Enhanced RT**

As a radiobiological concept, the ionizing radiation-killing potency is mainly dependent on the oxygen level inside the exposed tissue. However, the tumoral tissue microenvironment is naturally hypoxic, therefore, the tumoral cells are more insensitive toward radiation compared to healthy cells, causing a harshly declined therapeutic efficiency. The mild hyperthermia can accelerate blood flow within the tumor tissue to improve intratumoral oxygenation, which alleviates hypoxia-mediated radioresistance, triggering elevated RT efficiency. Hence, to realize the synergistic effect, the target tissue should be exposed to ionizing radiation after photothermal therapy. Furthermore, the thermal effect is able to successfully inhibit the sublethal damage repair induced by XRT, offering a higher PTT/RT co-killing effect. These mechanisms behind PTT-enhanced RT can make a beneficial link between photothermal and ionizing radiation for introducing a new therapeutic strategy of PTT/RT, known as thermoradiotherapy. To date, a number of studies revealed a synergistic therapy effect of PTT/RT in the presence of Bi NPs (**Table** **5**). Thanks to the photo-to-thermal conversion and radiosensitization capability of Bi, the co-delivery of Bi NPs and NIR laser/X-ray irradiation can create a synergistic PTT/RT effect to completely ablate the tumor.

**Table 5.**List of developed Bi nanocompounds for synergistic PTT/RT

|  |  |
| --- | --- |
| **Types** | **Representatives** |
| Bi NPs[32] | Bi nanocrystals |
| Bi2Se3 NPs[8, 28, 46-48] | MnSe/Bi2Se3, PFC/Bi2Se3, Bi2Se3/silica, FeSe2/Bi2Se3 |
| Bi2S3 NPs[29, 37, 67] | MnS/Bi2S3, MOS2/Bi2S3 |
| Cu3BiS3 NPs[20, 46] | Cu3BiS3 |

Colony formation assay results clearly exhibited that co-treatment of BSA-Bi2Se3 NDs[29] and X-ionizing radiation resulted in a radiosensitivity and DNA strand breakage in cells, dependent on dose (**Figure** **13a**). A greater density of ɤ-H2AX foci upon a dose of 6 Gy X-rays + Bi2Se3 NDs caused serious DNA damage in 4T1 cells (Figure **13b**). The nontreated cell potency to self-repair of DNA damage induced by X-ray irradiation was found to be 3.66, which was reduced to a value of 2.0 in the presence of Bi2Se3 NDs. The parameters of SER and the quasi-threshold dose (*D*q) are introduced as vital indicators to evaluate radiation effectiveness. The required dose to reduce cell survival to 63% (*D*0) in X-ray treatment alone was 1.33 Gy, while co-treatment with the Bi2Se3 NDs triggered a significant cell-damaging effect with a *D*0 of 1.25 Gy; also, a decrease in the *D*q value from 1.58 to 0.92 Gy was measured (Figure **13c**). The reduction in all the parameters indicated a radiation/NDs co-killing effect, which induced a SER of 6%. Thanks to the high photothermal capability of Bi2Se3 NDs (η = 50.7%), the Bi2Se3 NDs and NIR laser irradiation cooperatively and completely killed 4T1 cells, following a laser power density- and NDs concentration-dependent manner. Despite the higher suppression capability of Bi2Se3/X-ray and Bi2Se3/NIR laser compared to those in the control, Bi2Se3, RT, and PTT, failed to completely eradicate the tumor. In contrast, a complete tumor ablation with no recurrence was achieved by the synergistic effect of BSA-Bi2Se3/PTT/RT (Figure **13d**).

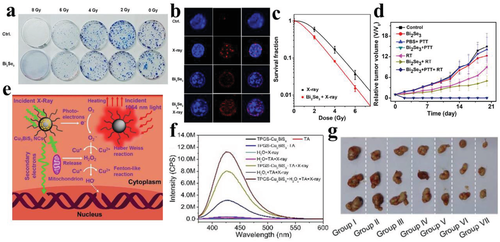
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Figure 13 a) Photograph of colony formation of 4T1 cells after different treatments. b) Images of 4T1 cell nuclei and c) their survival curves after different treatments. d) Tumor size in mice for various treatments. Reproduced with permission.[29] Copyright 2016, American Chemical Society. e) Sensitization mechanism induced by TPGS-Cu3BiS3 NCs. f) Fluorescence intensity in different solutions after different treatments. g) Photos of tumors for control, NCs, RT, laser, PTT, NCs + RT, and NCs + RT + PTT (from left to right). Reproduced with permission.[60] Copyright 2017, The Royal Society of Chemistry.

The cooperative mechanisms between Cu3BiS3 NPs[46] and NIR laser effectively improved the tumor oxygenation status (Figure 13e): in the first step, a high fraction of X-radiation produced a large number of the photoelectrons and Auger electrons mediated by a Bi-radiosensitizer. In the second step, the Cu+ ions located on the surface of d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS)-Cu3BiS3 underwent a rapid reaction with H2O2 molecules inside the tumor cells through the Fenton-like reaction to generate a very reactive type of HO• and Cu2+ ions:

(1)

In the third step, the heat arising from TPGS-Cu3BiS3 NCs upon NIR irradiation could greatly amplify blood flow in the tumor environment, causing oxygen-rich intratumoral tissue. The generated photoelectrons and Auger electrons reacted with the medium O2 molecules to form a bulky number of superoxide ions (O2•−):

(2)

Afterward, the formed O2•− are more willing to react with Cu2+ ions as a product of the Fenton-like reaction to achieve Cu+ ions:

(3)

On the other side, when superoxide (O2•−) reacts with H2O2 molecules, it will generate highly reactive HO• as follows:

(4)

As a result, the Cu+ ion-rich TPGS-Cu3BiS3 NCs surface served as a catalyst to speed up the decay of H2O2 inside cancerous cells, which generated HO• to cause higher cell death rates.

To evaluate the contribution of TPGS-Cu3BiS3 NCs during the production process of HO• through the Fenton-like reaction, the fluorescence intensity of TA-OH was measured. It is clear that the introduction of TPGS-Cu3BiS3 NCs significantly amplified the intensity of fluorescent rays emitted from TA-OH (Figure **13f**), which was largely attributed to the Cu+ ions during decomposition of H2O2 to beget HO•, triggering further lethal DNA damage of tumoral cells for enhanced RT efficiency. The expectable fluorescence intensity mediated by the TPGS-Cu3BiS3 NCs was measured under irradiation of X-rays in the presence/absence of 1064 nm. The PTT/RT synergistic effect was further confirmed by the reduced tumor weight of mice, which co-received NPs/PTT/RT (Figure **13g**).

Interestingly, a newly synthesized NP (PVP-Bi2Se3@Sec)[46] not only improved the sensitivity of tumoral tissues to NIR ionizing and radiation as a hyperthermia enhancer and radiosensitizer, but it also amplified in vivo protection of healthy tissues to X-rays as radioresistance (**Figure** **14a**). The mean tumor weight in the NIR + RT + PVP-Bi2Se3@Sec NPs group was lower than the other groups (Figure **14b**). This means that PVP-Bi2Se3@Sec-enhanced PTT and RT synergistically destructed tumors without recurrence. Additionally, PVP-Bi2Se3@Sec NPs progressively degraded. In this process, the selenium ions detach from the NPs and were released into the blood circulation system. This released selenium could improve glutathione peroxidase (GSH-Px) activity and enhance the removing ability of excess free radicals, consequently improving the immune-response and diminishing RT induced side effects.

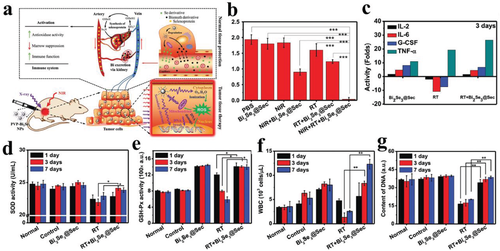
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Figure 14 a) The mechanism of normal tissue protection and tumor therapy of PVP-Bi2Se3@Sec NPs. b) Average weight of mice tumors treated by various agents. c) Cytokine concentrations in serum for different groups of mice. d) SOD and e) GSH-Px activity in serum. f) WBC counts and g) marrow DNA content for mice who received different treatments at different time points. \*\*\*p < 0.001, \*\*p < 0.01, and \*p < 0.05. Reproduced with permission.[46] Copyright 2017, Wiley-VCH.

Cytokines, as an important component of the immune system, play a major role in cell signaling. PVP-Bi2Se3@Sec NPs treatment led to higher levels of interleukin-2 (IL-2), interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF), and tumor necrosis factor alpha (TNF-α) in serum (Figure **14c**). In contrast, a downregulated secretion level of IL-2, IL-6, and G-CSF was found after X-ray radiation, which obviously decreased immune system function. While for samples treated with RT + PVP-Bi2Se3@Sec NPs, the serum collected for cytokine analysis and, interestingly, the level of TNF-α for the group was significantly higher in comparison to PVP-Bi2Se3@Sec NPs or RT alone groups. These results demonstrated that PVP-Bi2Se3@Sec NPs could improve immune system function by increasing cytokine recovery. Furthermore, SOD (Figure **14d**) and GSH-Px levels (Figure **14e**) decreased after X-ray irradiation, while administration of PVP-Bi2Se3@Sec NPs resulted in a recovered level of SOD and GSH-Px activity. This phenomenon offered enhanced antioxidant tolerance to improve the recovery rate of radiation-induced damage. These results, along with the recovered level of white blood cells (WBCs) (Figure **14f**) and no obvious change in marrow DNA content (Figure **14g**) in RT + PVP-Bi2Se3@Sec group, suggested that PVP-Bi2Se3@Sec NPs could modulate immune system action and improve hematopoietic activity. Therefore, the PVP-Bi2Se3@Sec NPs could diminish ionizing radiation adverse effects through improvement of immunological system function.

MnSe@Bi2Se3 NPs[47] have served as an effective probe for hyperthermia-mediated improved RT efficiency (**Figure** **15a**). The weak fluorescence intensity of the hypoxia-specific Ir–PVP complex as a quantitative marker of oxygen showed a decrease in the degree of tumor hypoxia in the MnSe@Bi2Se3 NPs + NIR irradiation group (Figure **15b**). The effective increase of oxygenation status in the tumoral tissue after treatment with PEGylated-MnSe@Bi2Se3 NPs + NIR laser irradiation was confirmed through immunoperoxidase images of stained parts of the hypoxic tumor (Figure **15c**).

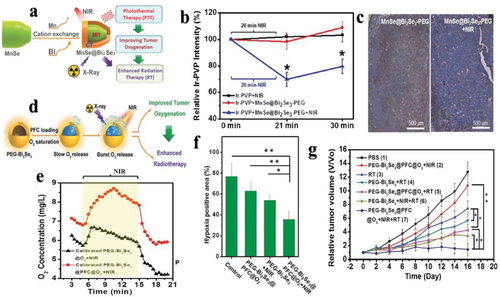
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Figure 15 a) The illustration of the different applications of theranostic MnSe@Bi2Se3 core–shell NPs for MR/CT imaging and photothermal/radiotherapy. b) The relative fluorescence intensities as a function of NIR irradiation time. c) Immunohistochemical images of mouse tumor cells treated with the synthesized nanocomposite in the presence/absence of NIR irradiation. Reproduced with permission.[47] Copyright 2015, Wiley-VCH. d) Schematic of the PEG-Bi2Se3@PFC@O2 structure for loading oxygen, which can be released more under NIR laser irradiation. e) O2 concentration in solutions of PEG-Bi2Se3@O2 or PEG-Bi2Se3@PFC@O2 under NIR laser irradiation. f) Level of tumor hypoxia for different groups. g) Relative tumor volume of mice during 16 days after different treatments. \*\*p < 0.01 or \*p < 0.05. Reproduced with permission.[48] Copyright 2016, Wiley-VCH.

To date, several effective techniques have been developed to improve RT efficiency in the tumoral microenvironment hypoxia. It is thought that pumping oxygen gas with high-pressure into the tumor may be an efficient strategy to elevate oxygenation content. But, it is not always practical, as almost all tumor sites are located in inner organs, which are poorly exposed to external oxygen. Nanotechnology is a promising way to fabricate oxygen-loaded nanocarriers for oxygen delivery. For this propose, thanks to the successful RT and PTT function of Bi2Se3 NPs in a previous study, Bi2Se3 NPs were modified to deliver oxygen into hypoxic tumoral tissues. The hollow PEGylated Bi2Se3 NPs[48] could successfully encapsulate temperature-sensitive perfluorocarbon (PFC) to serve as an oxygen reservoir (Figure **15d**). PFC is an inert, colorless and nontoxic liquid with high affinity for oxygen, therefore, it has been used as an oxygen carrier with a high capacity to dissolve oxygen. Based on this, PFC-loaded hollow PEG-Bi2Se3 (PEG-Bi2Se3@PFC) could gradually release oxygen in a hypoxic microenvironment and serve as an oxygen reservoir. In the PEG-Bi2Se3@PFC@O2 group, the loaded oxygen molecules were gradually released within the tumor site, which led to remarkably higher radiation-induced DNA damage in cancerous cells than that of the PEG-Bi2Se3 group. Most importantly, co-treatment of PEG-Bi2Se3@PFC@O2 and NIR light resulted in instant tumor oxygenation (Figure **15e**), due to a burst release of oxygen from NPs, which could be a promising way to decrease hypoxia-associated RT-resistance in tumor cells (Figure **15f**). Therefore, RT treatment with PEG-Bi2Se3@PFC@O2 under NIR light could significantly decrease tumor growth, which was unachievable by PTT or RT monotherapy (Figure **15g**).

### **4.2.3 Photothermal-Enhanced PDT**

As an invasive cancer therapy modality, PDT is activated under light irradiation to alternatively ablate tumoral tissue. Its cell death mechanism directly relies on the cytotoxicity triggered by the selective generated ROS inside the tumor cells exposed to light laser. Therefore, its treatment efficiency is mainly dependent on three factors of light: the fraction of the absorbed light either by itrogenous or exogenous (photosensitizer (PS)) agents and the content of oxygen within the tissue. The intracellular introduction of PSs into the body is considered as an effective strategy to amplify PDT efficiency.

As previously described, mild hyperthermia can successfully improve cell membrane permeability to achieve higher intracellular uptake of exogenous agents, namely, the PS-incorporated nanocarriers. It is clear that the concentration of intracellular PS determines the generated ROS amount and, consequently, PDT efficiency. Additionally, mild hyperthermia accelerates the blood flow to enhance oxygenation inside the tumor vascular. The PS absorbs a large fraction of light and deposits its energy to molecular oxygen (O2) in the oxygen-enriched tumoral environment to form a highly reactive singlet oxygen 1O2. In other words, the oxygen molecules are largely consumed in PDT, which in turn, exacerbates the hypoxia status in the tumoral cell environment, therefore, not only inhibiting the higher treatment efficiency of PDT, but also promoting tumoral tissue recurrence. As a result, the co-treatment of PDT and PTT yield a strong synergistic treatment effect in hypoxic cancerous cells, owing to hyperthermia-enhanced intracellular ROS generation.

Producing ROS upon laser exposure in an aqueous solution with no depletion in molecular oxygen is an approach to suppress the PDT-induced hypoxia. In this regard, bismuth tungstate (Bi2WO6) NPs[49] were developed. The ROS-indicator green fluorescence upon laser irradiation was observed in the cells incubated by indocyanine green (ICG) as a conventional PDT agent, W18O49 NPs and Bi2WO6 NPs, with superior fluorescence intensity for Bi2WO6 NPs (**Figure** **16a**). A red fluorescence in the ICG and W18O49 NP groups indicated the oxygen consumption to generate ROS and hypoxic environment post-PDT; in contrast, no such hypoxic condition was observed in the case of Bi2WO6 NPs with superb ROS generation capability.

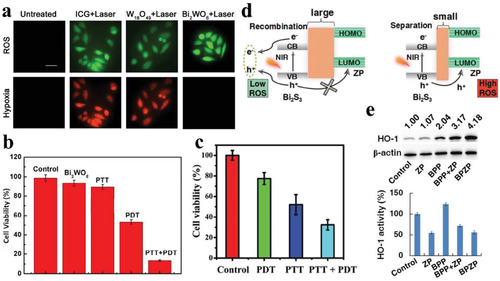
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Figure 16 a) Stained HeLa cells for illustration of intracellular ROS and hypoxia. b) Relative HeLa cell viabilities after different treatments with Bi2WO6 after 24 h of incubation. Reproduced with permission.[50] Copyright 2017, Wiley-VCH. c) Phototherapeutic effect of BFO NPs toward HepG2 cells. Reproduced with permission.[50] Copyright 2018, Wiley-VCH. d) Different charge transfer process between Bi2S3 and ZP dependent on their distances under NIR laser irradiation. e) HO-1 expression and activity of 4T1 cells treated with BPZP for 24 h with 808 nm laser irradiation. Reproduced with permission.[51] Copyright 2019, Wiley-VCH.

The co-activation of heat and ROS generation capability of Bi2WO6 NPs triggered HeLa cell death (87%) over the sum of those for PTT and PDT alone (Figure **16b**).

Bi2WO6 NPs not only have photothermally mediated antitumor activity but with the highly active surface for photocatalytic oxidation reactions, generate •OH radicals. No consumption of molecular oxygen in this process significantly overwhelms the PDT induced hypoxia.

In line with tungsten, the desired level of ROS can be produced from synergistic mechanisms between Fe3+ and Bi3+ ions (BFO NPs) immediately after laser irradiation.[50] H2O2 potentially can react with Fe3+ which produces •OH via a Fenton-like reaction. In the meantime, the reaction with oxygen molecules of unpaired electrons can generate superoxide ions in mitochondria. Also, •O2− can react with •OH to form 1O2. It has been shown that the BFO NP-mediated single PTT or PDT antitumor effect is lower than combined PTT/PDT (Figure **16c**), signifying that the combination of the single matter of BFO NPs with NIR laser irradiation leads to a significant cooperative antitumor activity.

It has been reported that the Bi2S3 NRs showed superb photothermal performance after NIR exposure. Further, Bi2S3 NRs[51] can be used for potential NIR-activated synergistic PTT/PDT due to the generation of free electrons, which form hydroxyl and superoxide radicals upon reaction with water and oxygen, respectively. Nevertheless, there are two major obstacles for achieving the desired efficiency of PDT: decreased ROS production due to high-speed electron–hole recombination in Bi2S3 NRs, and significant tumor cell protection against the elevated level of ROS stress due to innate antioxidant mechanisms. To address these issues, a type-II heterostructure was developed from a combination of zinc protoporphyrin IX (ZP) with Bi2S3 NRs. This heterostructure can potentially aid electron–hole separation to generate ROS (Figure **16d**), and also, it can inhibit OH−1 activity which is the major endogenous scavenger of ROS in the innate antioxidant defense system of the cell (Figure **16e**).

However, Cheng et al.[52] recently developed a simple technique to prepare AgBiS2 NPs with dual PTT and PDT performance, compared with the above reported Bi-based NMs. Interestingly, AgBiS2 NPs have shown remarkable antibacterial properties, which can be very promising since bacterial infection is one of the most important issues in tumor therapy.

# 5 Nanotechnology for Trimodal Synergistic Therapy

As mentioned, a combination of two therapeutic agents for bimodal synergistic therapy increased antitumor effects more than the corresponding monotherapy. However, based on evidence, the efficacy of antitumor treatments can be further improved via a trimodal synergistic therapeutic approach. In this approach, a cooperative improvement among three types of therapeutic agents within a single nanoagent can simultaneously target different pathways in cancer cells. Furthermore, a trimodal synergistic therapy can diminish unwanted side effects through a reduction in dose of the administered therapeutic agents. In the case of Bi NPs, only two types of trimodal synergistic therapies of PTT/PDT/CTX and PTT/RT/PDT have been reported (**Figure** **17**).

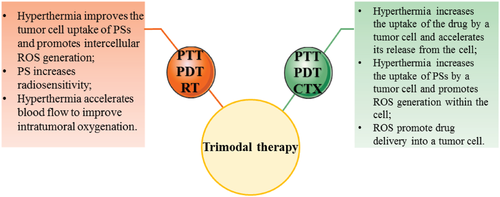
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Figure 17 The interconnection between PTT, PDT, and RT.

## 5.1 PTT/PDT/RT

The incorporation of PS and PT agents into a single nanosystem can provide a combination of light laser-triggered PDT and PTT, effectively, if they are elements with a high atomic number. A more successful therapeutic effect can be concurrently achieved by co-treatment with X-ray-triggered RT. PTA-mediated heat is able to improve the fraction of swallowed nanocarriers by tumor cells and accelerate intratumoral blood flow, which substantially enhances oxygen content within a hypoxic tumor microenvironment to achieve higher DNA damage induced by the PDT and RT antitumor effect via further generation of intracellular ROS and cell radiosensitization. The PDT relies on a series of light-triggered chemical reactions to generate ROS (largely singlet oxygen) which consequently initiates DNA damage in tumoral cells. In contrast, RT produces ROS (largely free radicals) under energetic ionizing radiation to breakdown DNA strands. Therefore, for PDT and RT, nuclear DNA is considered as a dominant target, hence co-utilizing two anticancer modalities offers a potential synergistic therapeutic effect to suppress tumor growth via lethal cell damage. As a result, PTT-enhanced RT/PDT can lead to the combination of PTT/PDT/RT modalities together, which is effective in comparison with bimodal and monotherapies.

By co-loading BiOI and Bi2S3 NPs[53] within a single nanocarrier, a triple-treatment based on radio/photodynamic/photothermal could be achieved (**Figure** **18a**). Taking into account that BiOI is considered as a photocatalytic semiconductor, not only can it produce an electron–hole pair, but it can also serve as the radiosensitizer to enhance the deposited radiation dose and subsequent therapeutic effect. Since highly reactive species, such as •OH and O2•−, are almost the dominant products of the reaction between ionizing radiation and water or oxygen molecules inside the exposed cell, BiOI NPs are able to work as a photosensitizer promoted by X-ray to yield a larger number of reactive species for an enhanced killing effect. With the heterogeneous growth of Bi2S3 on the BiOI surface to form BSA-coated BiOI@Bi2S3 NPs, abbreviated as SHNPs, they excellently convert photo-to-thermal energy and improve the oxygen content in the hypoxic tumor environment, effectively resulting in cancer cell death mediated by a synergistic laser radiation/X-ray co-killing effect. Also, SHNPs could significantly reduce the recombination tendency of electron–holes and improve the number of electron–hole pairs, comprising a greater catalytic activity under exposure to light.

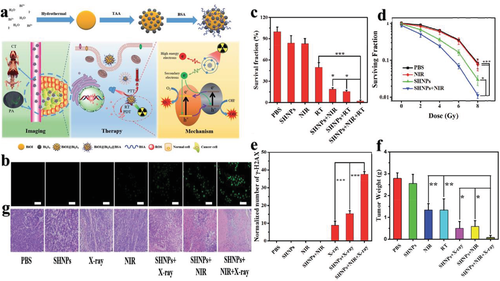
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Figure 18 a) The BSA-coated BiOI@Bi2S3 semiconductor heterojunction NP synthesis process and the related mechanism for radio/photodynamic/photothermal therapy. b) Level of ROS after different treatments of BEL-7402 cells. Scale bar is 100 µm. c) Cell viability and d) the colony formation curves for treating the cells after different treatments. e) γ-H2AX foci number of the treated cells. f) The weight and g) H&E images of the tumorous mice after various treatments. \*\*\*p < 0.001, \*\*p < 0.01, and \*p < 0.05. Reproduced with permission.[53] Copyright 2017, Wiley-VCH.

Under X-ray radiation, the SHNPs followed an oxygen-free mechanism ablate tumoral tissue through a combination of RT, PDT and PTT. For groups treated by SHNPs, fluorescence images revealed a stronger fluorescence signal of oxygen enrichment and tumor hypoxia for the alleviation induced by co-treatment with NIR + X-ray, where weaker fluorescence intensity was observed for the SHNPs + X-ray group based on the X-ray triggered photosensitizer property of BiOI NPs (Figure **18b**). The SHNPs + NIR + X-ray treatment decreased the colony forming capability of BEL-7402 cells to 2.1% with an SER of ≈1.79 (Figure **18c**,**d**). The ɤ-H2AX staining confirmed the hypothesis of the double DNA strand break as a major target in synergistic PTT/RT/PDT therapy (Figure **18e**). Correspondingly, upon sequential exposure of tumor tissue to a NIR laser and X-ray, SHNPs produced a significant synergistic PTT/PDT/RT effect for almost complete suppression of tumor proliferation (Figure **18f**), largely inducing tumor annihilation. The X-ray or NIR single treatment was able to induce weak tumor regression, while their treatment efficiency significantly improved when mediated by SHNPs, but the tumor growth suppression capability of SHNPs + X-ray or SHNPs + NIR still was not as successful as their synergistic therapeutic effect. Furthermore, the highest degree of necrosis and apoptosis in cells was observed by staining tumor tissue of those treated with SHNPs + NIR + X-ray (Figure **18g**), while the monotherapy-induced a partial therapeutic effect within the tumoral tissue, further indicating the unique effectiveness of PTT/PDT/RT triple therapy.

## 5.2 PTT/PDT/CTX

The incorporation of PS, drug, and PTCA into a single nanostructure potentially can be used to develop new combination therapies from conventional monotherapies such as CTX, PDT, and PTT. For example, tumor cell uptake of nanocarriers and the release of drugs and PS into the cytoplasm remarkably enhanced by the heat produced from light-activated PTA. Subsequently, elevated probabilities of drug/ROS-induced DNA damage improved CTX and PDT efficacy. Also, ROS produced upon PDT can elevate the intracellular release of drugs by dodging endosomal uptake. Based on this, PTT-enhanced CTX/PDT and PDT-enhanced CTX can be used to develop synergistic trimodal PTT/PDT/CTX combination therapy, with higher efficiency than their bimodal combinations.

As reported in Section **4.2.3**, Bi2WO6 NSs[54] can produce both heat and ROS in response to large wavelength ranges due to the narrow bandgap (1.15 eV). Further, Bi2WO6 NSs possess high drug loading capacity due to the high surface to volume ratio resulting from its corrugated lattice arrangement (**Figure** **19a**). Owing to these features, Bi2WO6-DOX-PEG NSs can be used for the simultaneous utilization of CTX, PDT, and PTT, which potentially generate better anticancer effects in vitro and in vivo. Bi2WO6 NSs were able to elevate heat (Figure **19b**) and generate 1O2 (Figure **19c**) for PTT and PDT immediately after 808 nm laser exposure. These features of Bi2WO6 NSs were not weakened by a particularly high DOX loading capacity (81.3% in weight). In contrast, the elevated heat could increase tumor cell uptake of Bi2WO6 NS-DOX (Figure **19e**) and facilitate the release of DOX from Bi2WO6 NSs (Figure **19d**), which results in more cytotoxicity (Figure **19f**).

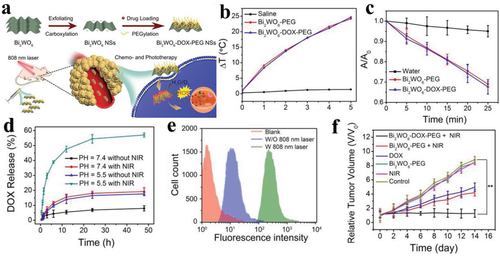
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Figure 19 a) The Bi2WO6-DOX-PEG NSs synthesis process and the related mechanism for radio/photodynamic/photothermal therapy. b) Temperature change curves of Bi2WO6-DOX-PEG NSs irradiated for 5 min. c) Detection of generated ROS using DPBF as a chemical probe in the presence of Bi2WO6-DOX-PEG with 808 nm laser irradiation. d) Controlled DOX release behavior of Bi2WO6-DOX-PEG NSs under various pH values with or without NIR laser irradiation at 37 °C. e) Flow cytometry analysis of DOX fluorescence in L929 cells after incubation with Bi2WO6-DOX-PEG NSs, with or without 808 nm laser irradiation. f) The relative tumor volumes of mice treated after various treatments. \*\*p < 0.01. Reproduced with permission.[54] Copyright 2018, Elsevier.

# 6 Conclusions and Future Prospects

Nowadays, a vast range of metallic NPs have emerged as diagnostic probes and therapeutic vehicles as well as probes for various biomedical applications. Recently, Bi metal with superior properties has been widely used as an effective therapeutic agent to successfully ablate tumoral tissues by inducing toxicity as a result of external stimuli responses. This review presents a comprehensive view on recent advances in tumor targeting and therapeutic applications of Bi-based NMs. Several therapeutic approaches including monotherapy, bimodal synergistic therapy, and trimodal synergistic therapy have been introduced.

Biosafety is an important prerequisite for the clinical translation of Bi-based NMs. Due to increasing biological applications and innate characteristics of Bi-based NMs (which directly interact with cellular components as organelles, plasma membranes, DNA, and proteins), the cytotoxicity of these Bi-based NMs should be addressed before any potential clinical translation.[55] The majority of previous studies have provided evidence regarding low or no cytotoxicity of Bi-based NPs even at high doses. For example, it has been shown that Bi2S3 NPs have no cytotoxicity against several in vitro systems such as U937 and HepG2 (20 × 10−3 m), and 4T1 and J774 cells (15 mg Bi2S3 mL−1).[31] Bi2O3 NPs are also well tolerated in murine periodontal ligaments and rat osteosarcoma cells.[56]

Further, the safety of several Bi-based NMs have been exhibited in a review by Cheng and Zhang.[57] In contrast, Liu et al.[58] have found that Bi2S3 NPs induced more cytotoxicity in human embryonic kidney 293 cells (HEK293) when compared to other cell lines such as HepG2, umbilical vein endothelial cells (HUVEC), and lung adenocarcinoma A549 cells, and this effect was attributed to the release of Bi ions. In line with these findings, urea and creatinine levels were elevated during the first 3 days after administration of Bi2S3 NPs, showing a more specific cytotoxicity effect to kidney cells.[58] The possible mechanism of nephrotoxicity may be due to autophagy associated pathways induced by bismuth via upregulation of microtubule associated protein light chain 3 (LC3) in Bi2S3 NP-treated HEK293 cells.[58] Based on these findings, a comprehensive cytotoxicity and safety profile for Bi-based NMs is not available and the probable mechanisms need to be further clarified. Here, we provide the current state of art and potential suggestions for future clinical translation of the therapeutic strategies induced by Bi-based NMs.

First, to benefit from the therapeutic effect of Bi nanoprobes, adequate accumulation of the nanoprobes inside the tumor is necessary. Some key points to address this issue are described as follows:

* The type of surface modifier has a large contribution to the blood retention time and tumor accumulation fraction of NPs. Despite the widespread use of PEG to reduce RES clearance of Bi NPs, RBC membrane coatings have caused superior blood circulation of Bi NPs. Therefore, “personalized nanoprobes” using a patient-extracted RBC membrane can be a promising strategy to avoid triggering the immune system response and also for inducing a large accumulation of Bi NPs within the tumor via the EPR effect.
* Co-integration properties using the EPR effect and renal clearance can be achieved by reducing the core size of Bi NPs below FST and minimizing serum protein binding with the surface of Bi NPs to reduce long-term toxicity and enhance tumor accumulation, respectively.
* As an active targeting approach, the attachment of targeting ligands (such as RGD, LyP-1, folate, and Tam) leads to greater uptake of Bi NPs into the tumors, compared with passive targeting.
* In a novel manner, “molecular Bi nanoprobes” can be designed to incorporate the renal clearable behavior of small molecules with the EPR effect of conventional NPs. These molecular nanoprobes exploit the advantages of small molecules in demonstrating a short half-life in the body, low accumulation in nontarget organs, and large renal clearance efficiency; profitably, they also show some characteristics of conventional nanoprobes like long circulation time and greater uptake into the tumor specifically. Therefore, an efficient nanosystem based on Bi should at least be composed of two components—a Bi NP as the core and a surface modifier and tumor targeting ligand as a shell, which possesses good biocompatibility, high tumor targeting accumulation capability, and easy biodegradability.

After reaching suitable accumulation of NPs at a tumor site, the limited efficiency of a single anticancer drug and the tumor heterogeneity is another challenge that needs to be solved. It is obvious that co-delivery of a multidrug with different therapeutic effects to the tumor can further mitigate the MDR of cells, leading to a more successful CTX. Here are the major potential characteristics of Bi-based NPs to enhance CTX efficacy:

* Bi NPs have shown a promising capability as an effective delivery system for DOX with large loading capacity, which not only overcomes MDR but also improves the chemotherapeutic effectiveness with no inevitable systemic toxicity, due to prolonged blood circulation half-life and the tumor-specific distribution of the DOX-loaded Bi nanovehicle.
* Thanks to the effective storage and sustained release of a chemotherapeutic drug loaded inside a Bi nanocarrier, it may be possible that the Bi NPs, especially their mesoporous type, also serve as carriers of therapeutic genes to develop a Bi NP-based genetic therapy.
* Most importantly, the modification of a Bi nanocarrier surface with positive charges may allow it to form complexes with the backbone of siRNA as well as further intracellular uptake due to electrostatic absorption of the cell membrane with a negative charge.

Besides CTX, ionizing radiation is another monotherapy approach. Despite the efficient role of Bi as a radiation sensitizer to amplify X-ray radiation dose, tumor cell hypoxia highly increases their resistance to ionizing radiation damage in comparison to healthy cells. Bi-based NPs can be helpful to overcome this limitation as described here:

* By engineering Bi NPs with a radiation-excited S-nitrosothiol (R-SNO) group, an on-demand radiation enhancement agent can be designed that not only increases the severity of radiation damage through both mechanisms of increasing the Bi-mediated dose and reducing the NO-mediated hypoxia, but also controls the release of NO through a radiation dose-dependent manner to efficiently suppress hypoxic tumor growth.

Another monotherapeutic approach that showed a great deal of hope is the photo-to-thermal conversion capability of Bi-based NPs, which resulted in the successful treatment of malignancies in vitro and in vivo. In this regard, some excellent applications and potential future approaches are mentioned as follows:

* The in situ formation of gold NDs on the surface of bismuth sulfide caused a higher photothermal performance than Bi2S3 NRs.
* The 3D crumpled-paper-like nanosheets forming the Bi2S3 nanoflowers acted as a high potential absorber of NIR light for improving PTT efficiency.
* By loading of some proteins—like HSP70, PEI, BAG3, and Twist—into Bi nanocarriers, the resulting siRNA-Bi NPs can inhibit the heat shock response of cancer cells; therefore, the downregulation of corresponding protein expression can decrease the thermal resistance of cancer cells for an improved photothermal effect.

The complex network of different pathological pathways and heterogeneity of tumors limits the clinical application of current monotherapeutic strategies. Considering the reported studies, bimodal therapies have presented superior success in the treatment of cancer tissues than corresponding monotherapies. However, future studies to obtain maximum therapeutic gain and minimum side effects on healthy tissues should be designed on multifunctional Bi NPs:

* For maximizing synergistic CTX/RT effects, the loading of Bi nanocarriers with radiosensitizing drugs such as Tirapazamine (TPZ), PTX, Dtxl, and cisplatin (CDDP) can be more effective to achieve both radiosensitization and ratio enhancement.
* While Bi NPs can effectively thermally ablate tumors, the encapsulation of certain types of siRNAs into a Bi nanocarrier can realize the combination of GT/PTT and cooperatively improve PTT efficacy by silencing the expression of heat proteins.

The four representative types of bimodal synergistic therapies mediated by Bi NPs demonstrated the greater therapeutic effectiveness over any monotherapy in addressing common tumor troubles such as MDR, hypoxia and metastasis. Furthermore, the effective model of Bi-mediated bimodal therapies, although unreported, and the underlying synergistic mechanisms are shown in **Figure** **20**[59] for future research directions to design Bi NPs and translate them for clinical applications.

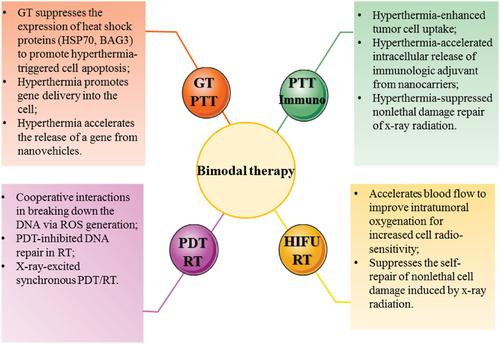
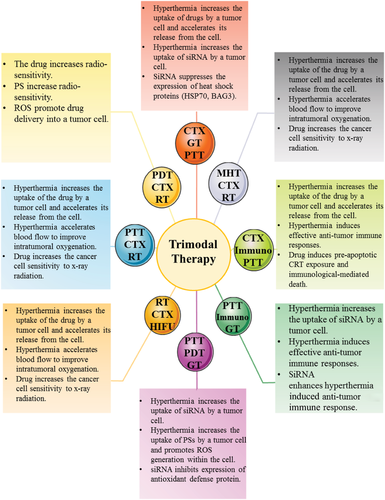
[](https://onlinelibrary.wiley.com/cms/asset/83b94ba2-64e0-4926-9228-e04117a776a1/adhm201901695-fig-0020-m.jpg)

Figure 20 Bimodal therapy types and related mechanisms.

Considering the enhanced therapeutic efficiency of Bi NP-mediated synergistic PTT/PDT/RT triple combination over synergistic PTT/RT and PTT/PDT dual combination and their significantly greater properties than PTT, PDT, and RT monotherapies, the design of novel multifunctional Bi NPs to cooperatively combine trimodal therapy techniques due to their synergistic interaction can largely expand the use of multimodal synergistic therapy in the future. There are potential kinds of a trimodal synergistic therapy based on Bi NPs that have not yet been reported, and corresponding synergistic mechanisms are shown in **Figure** **21** to develop advanced Bi nanoprobes for future clinical applications.[59]

[](https://onlinelibrary.wiley.com/cms/asset/600439b0-136d-4d7d-aef6-7c5dcd3514fe/adhm201901695-fig-0021-m.jpg)

**Figure 21** Trimodal therapy types and related mechanisms.

To gain a maximized multimodal synergistic effect, the sequential use of several treatments should be shifted to their simultaneous use. Only if the therapeutic advantages of various treatments are assembled into a single nanoprobe, can therapeutic success be achieved. However, the accurate diagnosis of tumoral tissue is a prerequisite for effective cancer ablation. Bi NP-mediated multimodal imaging guidance offers a comprehensive snapshot with detailed biological information about the location, size and shape of the tumor for efficient treatment. Therefore, Bi-based nanoprobes should simultaneously assemble therapeutic and diagnostic methods within itself for image-guided multimodal therapy.

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# Conflict of Interest

The authors declare no conflict of interest.

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