Chapter 11

Nanomaterials for combined thermo-chemotherapy of cancer

Javier Idiago-López,^{a, b, #} Eduardo Moreno^{a, #} and Raluca M. Fratila^{a, b, *}

^a Institute of Materials Science of Aragón (ICMA – CSIC/University of Zaragoza), C/ Pedro Cerbuna 12, 50009, Zaragoza, Spain

^b Centro de Investigación Biomédica en red en Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Zaragoza, Spain

Corresponding autor: rfratila@unizar.es; rmfratila@gmail.com

[#] These authors contributed equally to the present work

Abstract

Despite significant advances during the last decades, chemotherapy still suffers from important drawbacks such as severe side effects, insufficient drug availability in the tumor tissue, and multidrug resistance. Thermal sensitization can potentiate the effect of chemotherapy, resulting in a more efficient cancer treatment. In this regard, the combined approach of hyperthermia (HT) and nanomaterials is especially promising, as nanotechnology offers the possibility to construct multifunctional nanoplateforms for active targeting, drug delivery and therapy. In this chapter, we discuss several examples from the recent literature on the use of magnetic and plasmonic (gold and carbon-based) nanomaterials for thermo-chemotherapy, highlighting the synergistic effects of the combined approach. We conclude with a short discussion on the future challenges in the field in order to address the translational aspects of this new potential therapeutic solution.

Keywords

Magnetic nanoparticles; Gold nanoparticles; Carbon-based nanomaterials; Cancer treatment; Thermo-chemotherapy; Synergistic effect

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11.1 Introduction

Chemotherapy is one of the three conventional methods for oncological treatment, together with radiotherapy and surgery. (Chabner and Roberts, 2005) Despite significant advances during the last decades, the effectiveness of chemotherapy is often diminished by its severe side effects and low selectivity for the tumor tissue, which translates into insufficient drug availability. Moreover, it has been shown that a high percentage of the patients submitted to conventional chemotherapy treatments develop multidrug resistance (MDR). (Shah and Schwartz, 2001; Gottesman, Fojo and Bates, 2002) Therefore, there is a dire need of developing new solutions to improve current chemotherapeutic treatments. In this sense, the combined approach of hyperthermia (HT) and nanomaterials is especially promising, as highlighted in this chapter with significant examples from the recent literature.

The use of heat in cancer treatment has a long history and can be traced back to the antiquity, thus being known and implemented well before our ability to diagnose cancer (Seegenschmiedt and Vernon, 1995; van der Zee, 2002; Glazer and Curley, 2011). Hyperthermia, in the medical setting, can be defined as the procedure of increasing the temperature of the tumor tissue. A direct destruction of the tumor through necrosis caused by irreparable damage of proteins and other relevant biomacromolecules can take place at higher temperatures ($T > 45-47^{\circ}\text{C}$), although the preferred term in this case is thermal ablation. (Chatterjee, Diagaradjane and Krishnan, 2011) The most common form of hyperthermia leads to a thermal radio- and chemosensitization of the tumor tissue at temperatures of $41-43^{\circ}\text{C}$. (Beik *et al.*, 2016) The latter result arises from the tumor-selective effect of HT in vivo, which can be attributed to several factors: heat-induced alterations of the tumor blood flow and tumor microenvironment, changes in cellular membrane permeability, accelerated DNA damage and inhibition of DNA repair mechanisms, protein denaturation events and immune stimulation. (van der Zee, 2002; Torres-Lugo and Rinaldi, 2013; Shetake *et al.*, 2016; Peeken, Vaupel and Combs, 2017) There is ample evidence that this thermal sensitization can potentiate the effect of radio- and chemotherapy, resulting in a more efficient cancer treatment. (Hildebrandt *et al.*, 2002; Wust *et al.*, 2002; Issels, 2008; Datta *et al.*, 2015)

The concept of “thermo-chemotherapy” was demonstrated in the 1970’s, when Hahn *et al.* reported that the cytotoxicity of two chemotherapeutic agents in tumor-bearing mice was significantly increased by 43°C hyperthermia induced by an external hot water bath. (Hahn, Braun and Har-Kedar, 1975) The authors proposed the term “synergistic effect” to account for the fact that cell survival was significantly lower than the one predicted on the base of an additive effect of hyperthermia and chemotherapy.

From a practical point of view, the means of inducing hyperthermia are not so straightforward. Traditional methods for applying hyperthermia include whole-body, regional, and local hyperthermia. Whole-body HT can be induced by hot water baths or blankets and is normally used for metastatic situations. Regional HT is based on perfusion of hot liquids, for example intraperitoneal perfusion of heated chemotherapeutics, or perfusion of the patient’s ex vivo pre-warmed blood into the artery of interest. (Chatterjee, Diagaradjane and Krishnan, 2011) Finally, local hyperthermia can be achieved with probes positioned as close as possible to the tumor (luminal HT), thermal seeds and metal applicators placed into the tumor (interstitial HT) or heating using radiofrequency, ultrasounds or infrared radiation (external HT). (Chatterjee, Diagaradjane and Krishnan, 2011; Torres-Lugo and Rinaldi, 2013; Beik *et al.*, 2016) All these methods suffer from several drawbacks. Whole-body HT usually lacks tumor specificity, resulting in collateral damage of the surrounding healthy tissue,

while regional HT can be rather invasive. Concerning local methods, external HT is the least invasive, but the “out-inside” heating can induce hot spots also in the healthy tissue. Luminal and interstitial HT have moderate to high invasiveness and their implementation is rather complicated and expensive. Additional challenges preventing a widespread clinical use of HT are related with the precise temperature monitoring and the homogenous heat delivery to the target tissue. (Peeken, Vaupel and Combs, 2017)

Nanotechnology can offer new solutions in the field of thermo-chemotherapy, capitalizing on the heat generation by magnetic and plasmonic nanoparticles. As solid tumors typically have a leaky vasculature and inefficient lymphatic drainage, resulting in the so-called “enhanced permeability and retention effect” (EPR), nanoparticles with suitable size (10-150 nm) can passively accumulate in the tumor tissue. (Petros and DeSimone, 2010) For active tumor targeting, nanoparticles can be functionalized with suitable ligands that recognize receptors overexpressed by cancer cells. Once accumulated in the tumor tissue, magnetic and plasmonic nanoparticles can generate heat in a controlled fashion, the “inside-out” heating direction minimizing the adverse effects of traditional hyperthermia. Moreover, chemotherapeutic drugs can be also included in the design of a multifunctional nanoplatform and released selectively in the tumor tissue, thus improving the drug availability and reducing side effects associated with systemic administration. For all these reasons, nanomaterials are perfect candidates to bring to life the “magic bullet” concept (describing the ideal therapeutic agent, capable of targeting and treating only the desired cells) in cancer therapy introduced by Paul Ehrlich already in the 1900’s. (Datta *et al.*, 2016)

In this chapter, we will focus on magnetic, gold and carbon-based materials as thermo-chemotherapeutic agents, providing examples in which a clear synergy between heat and chemotherapy was achieved. Although, to the best of our knowledge, no clinical trial has been conducted so far with nanomaterials using the combined thermo-chemotherapy approach, its potential is demonstrated by numerous *in vitro* and *in vivo* studies.

11.2 Magnetic nanoparticle-based thermo-chemotherapy

Magnetic nanoparticles (MNPs) have awakened much interest for hyperthermia treatment of cancer due to their ability to use the energy of an external alternating magnetic field (AMF) to generate heat through several mechanisms (a detailed discussion regarding heat generation by MNPs is provided in Chapter 1 of this book). Other interesting properties of MNPs include their ability to target cancer cells through functionalization with tumor-specific recognition agents, the possibility to obtain stable colloids for many drug delivery routes, their capacity to cross the blood-brain barrier; and the combination of MNP-induced hyperthermia (also called Magnetic Fluid Hyperthermia, MFH) and drug release. (Kumar and Mohammad, 2011)

Many different metallic materials have been used in the development of MNPs for hyperthermia therapy, including Mn, Fe, Co, Ni, Zn, Gd, Mg and their oxides. Iron oxide-based systems (magnetite Fe_3O_4 , maghemite $\gamma\text{-Fe}_2\text{O}_3$, ferrites $\text{M}_x\text{Fe}_y\text{O}_4$, where M is Co, Mn or Ni), are widely used, although some non-iron-based nanoparticles have been also reported for hyperthermia applications, for example gadolinium, calcium, and lanthanum complexes. (Kumar and Mohammad, 2011; Mahmoudi *et al.*, 2011) However, iron oxide-based nanoparticles (IONPs) are the most popular, due to their superparamagnetism below a certain size (around 27 nm), their biocompatibility and low toxicity, highlighted by their approval by the Food and Drug Administration (FDA) as Magnetic Resonance Imaging (MRI) contrast agents. (Krishnan, 2010; Wang, 2011)

In this section, representative examples of the use of magnetic hyperthermia in combination with chemotherapeutic drugs are presented, so as to illustrate the wealth of different strategies developed in the recent years. We will focus on the applications rather than on the synthesis of the MNPs, as this topic is extensively covered in Chapter 2. For the interested reader, there are many other literature revisions extensively covering the use of magnetic nanoparticle in cancer therapy. (Hervault and Thanh, 2014; Datta *et al.*, 2016; Karponis, Azzawi and Seifalian, 2016; Kang *et al.*, 2017)

11.2.1 Magnetic nanoplatform designs for combined hyperthermia and chemotherapy

Several types of nanoplatform designs have been used for nano-thermo-chemotherapy applications, the most common approaches being discussed as follows (Figure 11.1). A typical example includes inorganic magnetic cores – usually IONPs – coated with an environment-sensitive polymer. The anti-cancer agent can be loaded by weak interactions with the polymer (e. g. adsorption, van der Waals forces, hydrophobic effects), encapsulation or labile covalent bonding to the polymer. The release of the drug can be triggered by different stimuli, typically pH, temperature, enzyme concentration, redox potential, magnetic field or light intensity. (Wang *et al.*, 2017)

In the case of MNPs, a temperature raise is easily achieved by the application of an alternating magnetic field (AMF). As for the pH, tumor microenvironments have an acidic nature, with pH values ranging from 5.7 to around 7.2 (physiological pH is 7.4). (He *et al.*, 2013; Liu *et al.*, 2014) Also, some intracellular compartments such as endosomes and lysosomes have a low pH value, which can trigger the drug release, provided that nanomaterials are able to enter the cell. Another frequently used nanodesign consists in the encapsulation of MNPs in amphiphilic structures (i.e. micelles or liposomes) along with the chemotherapeutic drug either as well embedded or covalently linked. This arrangement eases the combination of hydrophobic and hydrophilic molecules and MNPs in a single nanostructure. Long circulation liposomes have been recently improved, increasing the circulation time of the drug in the body while encapsulated to enhance its deposition in the tumor *via* EPR effect or active targeting. Moreover, temperature-sensitive liposomes can be synthesized using specific lipids and polymers. (Hervault and Thanh, 2014) Another common type of nanostructure used for magneto-chemotherapy is represented by core-shell nanoparticles, composed by a metallic core (usually MNPs) coated with an inorganic shell. (Kumar and Mohammad, 2011) Further organic modifications are usually performed in order to provide or improve stability, biocompatibility and biological activity or specificity. This approach is interesting, as MNPs advantages can be combined with attractive properties of other nanostructured materials.

Figure 11.1 Different nanopatform designs for magneto-chemotherapy: a) Environment-sensitive polymer coatings for the release of drugs triggered by external stimuli; b) Magnetoliposomes containing entrapped or encapsulated MNPs; the drug can be attached either covalently or by encapsulation and its release is typically achieved using pH or temperature; c) Core-shell and shell-shell nanostructures, in which the inorganic shell can act as gatekeeper and prevent drug leakage in vivo.

11.2.2 In vitro and in vivo examples of magneto-chemotherapy

Taratula *et al.* reported a multifunctional nanosystem for combined magnetic hyperthermia-chemotherapy (Figure 11.1), in which IONPs were coated with poly(maleic anhydride-*alt*-1-octadecene) (PMAO) in order to ensure stability in aqueous solutions and with an additional layer of poly(ethyleneimine) (PEI) that provides amine groups for functionalization. (Taratula *et al.*, 2013) Polyethylene glycol (PEG) was used to stabilize the nanoparticles in biological media and to anchor the luteinizing hormone-releasing hormone (LHRH) peptide as targeting unit for LHRH receptors on ovarian cancer cells. Doxorubicin (DOX) was loaded to the IONPs via physical encapsulation in the polymeric complex (IONPs-DOX). DOX is a chemotherapeutic anthracycline drug that intercalates into DNA, inhibiting topoisomerase-II and avoiding DNA repair. Although it is used for the treatment of several cancers, it suffers from several side effects, including high cardiotoxicity. (Thorn *et al.*, 2011) The drug release profile as a function of the pH revealed a much more efficient release at acidic pH (5.5) than at physiological pH due to the protonation of the amine group of DOX molecules, resulting in a weakened interaction with the carboxyl groups of the polymer. Thus, the system offers the possibility of achieving a high DOX concentration at the tumor site, while preventing the release of the drug during its transport through the blood stream. Nanoparticle-mediated mild hyperthermia (40°C, temperature reached after the application of an AMF with $f = 393$ kHz and $H = 21.2$ kA/m) decreased the viability of A2780/AD ovarian cancer cells by 72%, while the chemotherapy treatment with the nanosystem resulted in a 27% decrease in cell viability (encapsulated DOX is less cytotoxic than free DOX). However, the use of DOX-loaded

nanoparticles combined with magnetic hyperthermia resulted in a 95% cell death, proving the synergistic effect of the two approaches using a multifunctional nanocarrier.

Fig 11.2 Surface engineering of IONPs-based nanocarriers for targeted delivery and thermo-chemotherapy. After polymer loading with DOX (A), IONPs were functionalized with PEG (B) and LHRH was conjugated to the distal end of PEG molecules (IONPs-DOX-PEG-LHRH) (C) Reprinted from International Journal of Pharmaceutics, 458, Taratula *et al.*, “Multifunctional nanomedicine platform for concurrent delivery of chemotherapeutic drugs and mild hyperthermia to ovarian cancer cells”, 169-180, Copyright (2013), with permission from Elsevier.

Pradhan *et al.* synthesized a dual pH- and thermo-sensitive drug delivery system based on mesoporous magnetite nanoassemblies encapsulated in thin lipid layers (LMMNA). (Pradhan, Srivastava and Bahadur, 2014) The lipid layers were assembled from 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (Chol) and 1,2-distearoyl sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG2000), resulting in temperature and pH-sensitive system. Doxorubicin was encapsulated in the MMNAs, while paclitaxel (TAX) was incorporated in the thin lipid layer (Figure 11.3a). Under acidic pH, the system presented a sustained release profile of the drugs (88:53 DOX:TAX ratio); the release could be enhanced by the application of an AMF. In vitro, cellular death upon application of the AMF ($f = 250$ kHz, $H = 23.4$ kA/m) in HeLa, MCF-7 and HepG2 cells incubated with DOX/TAX-LMMNA was significantly increased in comparison with the use of DOX/TAX-LMMNA alone (no AMF applied, Figure 11.3b), demonstrating the synergy between chemotherapy and magnetic hyperthermia.

Fig 11.3 a) Overall concept of the dual pH- and temperature-sensitive drug delivery system based on lipid-magnetic nanoassemblies (LMMNA). b) Efficiency of the cellular death induced by LMMNA in the absence and in the presence of an alternating magnetic field, demonstrating the synergetic effect of thermo-chemotherapy. Reprinted from Acta Biomaterialia, 10, Pradhan *et al.*, “PH- and thermosensitive thin lipid layer coated mesoporous magnetic nanoassemblies as a dual drug delivery system towards thermochemotherapy of cancer”, 2976-2987, Copyright (2014), with permission from Elsevier.

The work of Gogoi *et al.* is a representative case of the use of MNPs other than iron oxides, as both Fe_3O_4 nanoparticles and $\text{La}_{0.75}\text{Sr}_{0.25}\text{MnO}_3$ (LSMO) nanoparticles were encapsulated in liposomes. (Gogoi *et al.*, 2014) The authors designed a paclitaxel-loaded thermo-sensitive magnetoliposome containing a dextran-coated biphasic suspension of MNPs. Paclitaxel is an antineoplastic agent that inhibits the normal tubule dynamics needed for cell division and replication. The lipid bilayer was composed of 1,2-distearol-*sn*-phosphatidylcholine (DSPC) and cholesterol (8:2 ratio), while LSMO and iron oxide nanoparticles were loaded in a 10:1 ratio. In vitro, the viability of MCF-7 breast cancer cells exposed to the combinatorial treatment of AMF-induced heat ($f = 423$ kHz, $H = 10$ kA/m) and paclitaxel resulted in a 75.7% of cell viability decrease in 15 min. This result was significantly better than the ones obtained using single treatments (25.8% cell viability decrease using hyperthermia and around 50% for paclitaxel). More recently, the same group carried out an extensive in vivo biocompatibility and therapeutic evaluation of thermosensitive magnetoliposomes for combined chemotherapy and self-controlled hyperthermia. (Gogoi *et al.*, 2017) Relevant biochemical blood parameters, as well as liver and kidney functions were not altered significantly following intravenous administration of the magnetoliposomes, suggesting their suitability for in vivo applications. The therapeutic efficiency was evaluated using fibrosarcoma tumor-bearing mice exposed to different treatments (bare or paclitaxel-loaded magnetic liposomes, single or double dose administration). Hyperthermia treatment (three exposures, each at three days interval, $f = 250$ kHz, $H = 28$ kA/m, 30 min) with drug-loaded magnetoliposomes and double dose administration induced a decrease in tumor growth by 3.6-fold compared with the control untreated group. Afterwards, the presence of MNPs was studied in the excised tumor and vital organs of mice. No significant leaching or draining was detected, as the MNPs remained located mainly in tumor tissues and could barely be identified in the spleen. Thus, these results prove the feasibility of using different MNPs beyond iron oxides, or combination of various MNPs, without systemic toxicity and potential treatment efficacy.

Recently, Moorthy *et al.* described a combined magnetic and mesoporous silica nanocarrier, modified with a crown ether triad (CET). (Moorthy *et al.*, 2017) Mesoporous silica materials are very attractive materials owing to their high surface area, large pore size and volume, good biocompatibility and thermo-chemical stability. Furthermore, their surface can be easily modified. (Pang *et al.*, 2012; Hao, Li and Tang, 2017) All these properties make them perfect candidates for drug delivery. In combination with magnetic hyperthermia, they can constitute a promising vector for the merged thermo- and chemotherapy approach. The specific core-shell arrangement used in the study is composed of IONPs in the core as the magnetic agent for hyperthermia and mesoporous silica (MS) acting as shell, loaded with DOX as anti-cancer drug. CET molecules were used as gatekeeper units to avoid premature escape of the drug from the nanocomplex. They were integrated into the pore apertures by active metal-ligand complex coordination interaction. In this way, the apertures are effectively blocked with an approach that had not been explored before for stimuli-response applications. The heat generation in solution through magnetic hyperthermia was highly efficient, as an 18°C increase (from 27 to 45°C) could be achieved within 4 min ($f = 409$ kHz, $H = 8.6$ kA/m). However, taking into account the high concentration of MNPs used (10 mg/mL), this result must be treated with caution as such concentrations are highly unlikely to be reached in vivo, even if the MNPs are injected directly into the tumor. The release of the drug was investigated under different pH and temperature conditions. CET showed effective blockage at physiological pH, for no significant drug escaped from the nanostructure – only 0.5% was released at 37°C/AMF (pH 7.4). As the pH

decreases, simulating the tumor acidic conditions, CET units allow DOX to be released from the mesoporous silica. The capping was not dependent on the temperature, as negligible variations on the release efficiency were observed at room, physiological and hyperthermia temperatures (25°C, 37°C and 45°C, respectively). However, the exposure to an AMF significantly increased the drug release at low pH: 73.5% of DOX was released at pH 5.0 in the absence of AMF; the combination of pH 5.0/AMF resulted in 94.5% release in the same period of time. This combinational effect is probably due to the facilitation of the diffusion of DOX molecules from the MS channels, triggered by low pH and enhanced by the temperature gradient. The cytotoxicity and biocompatibility of the nanocarrier were evaluated in vitro using the human MDA-MB-231 hormone-independent mammary cell line. Core-shell nanoparticles without drug did not show any cytotoxic effects in the concentration range tested (20 – 100 µg/mL). When AMF was applied, a 23% cell death was observed at the maximum nanoparticle concentration used. DOX-loaded core-shell nanoparticles showed around 92% cell cytotoxicity when cells were exposed to AMF, compared to 86% cell death when no AMF was applied and to 65% viability loss with free DOX. Altogether, the results show a potential biocompatible system for combined thermo-chemotherapy.

Although not a classical example of combined thermo-chemotherapy, the work of Yoo *et al.* is worth of discussion as it addresses a main obstacle in hyperthermia treatments, namely tumor thermoresistance. (Yoo *et al.*, 2013) Heat shock proteins (Hsp) act as molecular chaperones to avoid denaturation and degradation of client proteins at temperatures higher than physiological. Thus, Hsp are considered to have a main role in the acquisition of thermoresistance by cancer cells after the application of consecutive cycles of hyperthermia. (Tabuchi and Kondo, 2013) Yoo *et al.* proposed a resistance-free apoptosis-inducing magnetic nanoparticles (RAIN) approach employing zinc-doped iron oxide magnetic nanoparticles ($\text{Zn}_{0.4}\text{Fe}_{2.6}\text{O}_4$) and functionalized with geldanamycin (GM), a benzoquinone ansamycin that inhibits Hsp90, via a thermally cleavable azo linker. A complete release of GM was achieved after 60 min at 43°C (AMF, $f = 500$ kHz, $H = 37.4$ kA/m), a temperature relevant for apoptotic hyperthermia. Interestingly, the amount of GM released under external heating in a water bath at 43°C was very low (< 7%), meaning that nanoscale heating can induce much higher temperatures in the vicinity of the nanoparticle surface and is critical for the cleavage of the azo bond. In vitro, using MDA-MB-231 breast cancer cells, the use of RAIN induced 100% apoptotic cell death after 70 min of exposure to the AMF with the previously mentioned parameters, demonstrating that inhibition of Hsp90 makes cells more sensitive to hyperthermia. Furthermore, in vivo RAIN-mediated apoptotic magnetic hyperthermia was successfully demonstrated in mice bearing MDA-MB-231 xenograft tumors. The nanoparticles were injected directly into the tumor and the animals were exposed to the AMF for 30 min. Changes in the tumor volume were then monitored during 14 days, showing that for the untreated control, an 8-fold volume increase was reached, while RAIN-treated tumors were eliminated at day 8. The use of bare MNPs induced an initial decrease in the tumor volume, but they grew back at day 10, reaching a 2.5-fold increase by day 14. Nevertheless, RAIN-treated tumors were eliminated at day 8. The concept could be extended to a combined magnetic hyperthermia - chemotherapy treatment including also a chemotherapeutic drug, in order to achieve maximum therapeutic efficiency.

11.3 Gold nanoparticles as thermo-chemotherapeutic agents

Plasmonic gold nanoparticles (AuNPs) display unique optical and photothermal features due to their localized surface plasmon resonance (LSPR), which consists in a collective oscillation of the electrons in the conduction band of gold. (Eustis and El-Sayed, 2006; Zhang, Wang and Chen, 2013; Smith *et al.*, 2015) Following absorption of incident optical radiation in resonance with the surface plasmon oscillation, heat is generated through electron-phonon interactions. Importantly, LSPR frequency and therefore the amount of heat produced by AuNPs can be tuned by changing their size, shape, aspect ratio and composition. For instance, Au nanospheres with diameters comprised between 10 and 100 nm display LSPR bands in the visible range (typically 517-575 nm). For biological applications, the use of near-infrared (NIR) light is advantageous because of its deeper tissue penetration and limited absorption by biological structures. Fortunately, significant advances in nanotechnology during the past two decades made possible the production of a wide variety of nanostructures possessing LSPR features that can be finely tuned from visible to NIR wavelengths (Table 11.1). (Shanmugam, Selvakumar and Yeh, 2014; Abadeer and Murphy, 2016; Li, Liu and Chen, 2017; Riley and Day, 2017)

Table 11.1 Structural features of gold nanomaterials for photothermal therapy discussed in this chapter. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) micrographs reproduced with permission from ACS Nano 2017, 11, 2403–2409. Copyright (2017) American Chemical Society.

Shape	Nanorods	Nanoshells	Nanocages
TEM/SEM
LSPR band	$\lambda_T = 500 \text{ nm}$ $\lambda_L = 650\text{-}850 \text{ nm}$	$\lambda = 780 - 800 \text{ nm}$	$\lambda = 800 \text{ nm}$

For more information on the synthesis and plasmonic properties of nanomaterials, the reader is directed to Chapter 4 of this book, while a detailed discussion on the cellular effects of optical hyperthermia can be found in Chapter 8. In this section, we discuss several examples of application of gold nanoparticle hyperthermia as cancer treatment in synergy with chemotherapy, either administered separately (Section 11.3.1) or as part of a multifunctional nanoplatform containing the chemotherapeutic drug and, in some cases, tumor targeting elements (Section 11.3.2).

11.3.1 Enhancement of the cytotoxicity of cancer chemotherapeutics with gold nanoparticle hyperthermia

Chan and co-workers reported one of the first proofs of concepts of combining nanoparticle heating with chemotherapy. (Hauck *et al.*, 2008) Using OCI AML3 human myeloid leukemia cells, the authors demonstrated that the dosage of cisplatin could be reduced from 75 μM in the absence of nanoparticle heating to 25 μM when used in combination with optical hyperthermia induced by gold nanorods (AuNRs) under the same laser irradiation conditions (810 nm, 1 W/cm² power density, 30 min). The synergistic effect of optical hyperthermia and chemotherapy was attributed to cellular

membrane damage induced by heat, probably resulting in an increased accumulation of the drug inside cells. A similar observation was reported by Fay *et al.* for an in vitro model of inflammatory breast cancer (IBC), considered as one of the most aggressive subtypes of breast cancer. (Fay, Melamed and Day, 2015) IBC is usually treated by a multimodal approach including surgery, radiotherapy, and chemotherapy using high dosages of anthracycline chemotherapeutics such as Doxorubicin, known for its severe cardiotoxicity. The chemosensitization of IBC tissue by hyperthermia could lower the drug dosage required, thus mitigating its adverse effects. Indeed, the authors demonstrated that gold nanoshells successfully induced chemosensitization on SUM149 IBC cells at subtherapeutic doses of doxorubicin (0.75 μM). This dose caused no significant cytotoxicity, as cells treated only with DOX showed $\sim 98\%$ viability; cells incubated with nanoshells and exposed to low-dose optical hyperthermia treatment (NIR light, 808 nm, 5.5 W/cm^2 power density, 3 min) revealed a viability of 85%. Finally, the viability of cells that received the combined treatment (photothermal therapy with Au nanoshells + DOX) was of $\sim 65\%$, demonstrating that the effect of the treatment was synergistic rather than additive (according to the work of Hahn *et al.*, the predictive additive effect of photothermal therapy and Dox would lead to a cellular viability of $\sim 83\%$, almost 20% higher than the one observed experimentally).

In the above-discussed examples, gold nanoparticles served only as heat inducers to enhance the effect of chemotherapy due to an increased cellular uptake of the drug resulting from alterations of the membrane permeability and fluidity and of the internalization kinetics as a result of heat exposure. As highlighted in the introduction of this chapter, a major advantage of using nanotechnology for hyperthermia applications is the possibility of decorating nanoparticles with targeting ligands that would promote their delivery in a selective manner only to the tumor tissue. For instance, the folate receptor (FR) is overexpressed in many types of cancer cells, thus being an attractive and highly specific target for delivery of therapeutics. (Sudimack and Lee, 2000; Parker *et al.*, 2005) Banu *et al.* showed that folate-functionalized spherical gold nanoparticles could promote a selective chemosensitization of cancer cells. (Banu *et al.*, 2014) Optical hyperthermia followed by cyclophosphamide (CPA) treatment resulted in a significant increase in the sensitivity of highly FR-positive MDA-MB-231 breast cancer cells to chemotherapy in comparison with MCF-7 cells that are FR-negative. Chemotherapy applied after the folate-targeted hyperthermia treatment resulted in a ten-fold decrease in the half-maximal effective concentration of CPA (EC_{50}) in MDA-MB-231 cells. Conversely, no significant difference in the EC_{50} values was observed for MCF-7 cells. The targeting approach facilitated a higher concentration of gold NPs inside MDA-MB-231 cells; the increased accumulation of Au NPs led to the production of considerably high amounts of heat, promoting the internalization of the drug due to the cellular membrane hyperfluidization.

11.3.2 Gold nanomaterials as multifunctional platforms for optical hyperthermia and chemotherapeutic drug delivery

In Section 11.3.1, we provided some representative examples of chemotherapy enhancement by optical hyperthermia, in which the drug was administered separately from the nanoparticles. However, the ideal nanomaterial for cancer thermo-chemotherapy would integrate the anti-cancer drug for a specific and controlled release at the action site. Moreover, for in vivo applications the incorporation of targeting ligands in the nanomaterial would ensure its increased accumulation in the tumor, improving the selectivity and efficacy of the treatment. To illustrate the broad range of

gold nanomaterials that can be used for these purposes, here we discuss several examples of gold nanoparticles of different morphologies.

Gold nanorods

Au nanorods possess interesting optical features arising from their anisotropy - a transversal LSPR band in the visible range (500 nm) and a longitudinal LSPR in the NIR region, which can be tuned (650 - 850 nm) by changing the aspect ratio. (Qin and Bischof, 2012; Shanmugam, Selvakumar and Yeh, 2014; Li, Liu and Chen, 2017) Nevertheless, for their biomedical applications two aspects need further optimization: their cytotoxicity, associated with the necessity to use cetyltrimethylammonium bromide (CTAB) for the synthesis of high quality NRs, and the cellular uptake, which has been shown to be highly dependent on their aspect ratio. (Yang *et al.*, 2016)

Gu and co-workers reported a multi-layer coated, DOX-loaded AuNR system for combined photothermal-chemotherapy in sarcoma tumor-bearing mice. (Chen *et al.*, 2014) The multilayer, assembled from different polyelectrolytes (PE) and bovine serum albumin (BSA), improved the biocompatibility of the AuNRs-PE-BSA system and allowed for the self-adsorption of DOX by electrostatic interactions (Figure 11.4). In vivo, after axillary injection, mice receiving AuNRs-PE-BSA-DOX and exposed to NIR irradiation displayed a much lower rate of tumor growth in comparison with mice treated with DOX alone (with or without NIR irradiation) or AuNRs-PE-BSA/NIR. The synergistic effect between chemotherapy and optical hyperthermia was attributed to the sensitization of the tumor tissue as a result of the heat.

Fig 11.4 a) Overview of the formation of AuNRs-PE-BSA-DOX and DOX release triggered by NIR light. b) Representative high-resolution transmission electron microscopy micrographs revealing the formation of the multilayers. c) Synergistic effect of thermo-chemotherapy induced by AuNRs-PE-BSA-DOX in S180 tumor-bearing mice. Reproduced from Biomater. Sci., 2014, 2, 996 with permission of The Royal Society of Chemistry.

Exploiting the fact that the mode of action of many anthracycline chemotherapeutics relies on their intercalation with DNA, Wang *et al.* designed a gold nanorod-based nanoplatform in which AuNRs were firstly coated with DNA via electrostatic interactions and then functionalized with DOX. (D. Wang *et al.*, 2014) The nanoplatform was tested in vivo using the 4T1 lung metastasis breast cancer model; a successful inhibition of the primary tumor growth, preventing the lung metastasis was observed using NIR light irradiation (655 nm, 1 W/cm², 2 min) and the AuNR-DNA-DOX system.

An example of multifunctional AuNR-based platform, integrating active tumor targeting and chemotherapy can be found in the work of Chen *et al.* (Chen *et al.*, 2015)

Gold nanorods were functionalized with a thermo-sensitive shell incorporating covalently linked doxorubicin and biotin as active targeting ligand. The release of DOX was triggered by NIR irradiation, the heat produced by the NRs inducing the cleavage of the thermolabile azo linker. In vitro, the multifunctional platform showed specificity for cellular lines overexpressing biotin receptors (SCC-7 squamous cell carcinoma and HeLa human cervical carcinoma), in contrast to biotin receptor-negative COS7 cells (African green monkey kidney fibroblasts). In vivo, upon intravenous administration in SCC-7 tumor-bearing mice, the AuNR accumulated selectively in the tumors due to their active targeting ability. Laser irradiation (808 nm, 1 W/cm², 3 min) induced a significant increase in the temperature of the tumors, reaching ~44°C irrespective of the time elapsed between the injection and the irradiation (two and 24 h, respectively). Not surprisingly, a synergy between heat and chemotherapy was observed in mice treated with the combined approach, resulting in a more efficient inhibition of tumor growth than the one achieved with each of the monotherapies: chemotherapy with free DOX, chemotherapy with AuNR-DOX and thermotherapy with AuNRs lacking DOX.

In another recent example of multifunctional nanoplatform based on AuNRs, Zhang and co-workers targeted tumor angiogenesis using polydopamine-coated AuNRs functionalized with arginine-glycine-aspartic acid (RGD) peptide, having a high affinity for $\alpha v \beta_3$ integrin. (Zhang *et al.*, 2016) The AuNRs included cisplatin as chemotherapeutic drug and iodine-125 for computed tomography imaging (RGD-IPT-PDA-GNRs). The multifunctional platform showed a high specificity for $\alpha v \beta_3$ integrin in vivo in mice bearing subcutaneous H1299 tumors (H1299 are $\alpha v \beta_3$ integrin-positive human non-small lung cancer cells). Although both thermal therapy alone (laser + RGD-I-PDA-GNRs lacking cisplatin) and thermal therapy combined with chemotherapy (laser + RGD-IPT-PDA-GNRs) efficiently eliminated the tumors three days after the laser irradiation, only the combined approach was successful at preventing tumor relapses.

Gold nanoshells

Au nanoshells are thin layers of gold surrounding a dielectric core and exhibiting a LSPR band in the NIR region. (Shanmugam, Selvakumar and Yeh, 2014) Several gold nanoshell-based nanocarriers have been tested as platforms for combined thermo-chemotherapy in vitro and in vivo. Tang and co-workers described a system consisting of a PEGylated (PEG = polyethylene glycol) gold nanoshell grown on silica nanorattle spheres (termed pGSNs) in such a way that it provided the desired plasmonic properties (LSPR peak wavelength at 824 nm) without completely masking the mesoporous structure of the nanorattles. (H. Liu *et al.*, 2011) Thus, the partial openness of the shells allowed for the sustained release of the chemotherapeutic drug docetaxel (DOC) loaded in the pGSNs under NIR laser irradiation. In vitro, a dose-dependent hyperthermia-mediated enhancement of DOC cytotoxicity was observed on human liver carcinoma cells HepG2, with an equivalent DOC concentration of 10 nM showing approximately 82% inhibition rate. In comparison, docetaxel alone had a 52% inhibition rate, while NIR laser irradiation killed only 40% of the cells. In vivo, ICR mice bearing subcutaneous tumors (H22 hepatoma) were treated with intravenously administered Taxotere (the commercial form of docetaxel) and pGSNs-DOC at a dosage of 20 mgkg⁻¹ DOC every four days for a total of 17 days of treatment. Tumor weights for the group treated with pGSNs-DOC were significantly lower than for the control and Taxotere groups, the treatment showing an overall average inhibition rate of ~85% (in contrast to the 57% inhibition rate achieved with Taxotere alone). In contrast to the previous example, Wang et al. proposed a leak-tight Au nanoshell drug carrier (Figure 11.5 a), in

which polymer nanovesicles (PNVs) with an inner aqueous core were loaded with DOX and coated with a shell of gold by in situ Au(III) reduction first to gold seeds (GS@PNV) and then to a continuous gold nanoshell (GNS@PNV). (L. Wang *et al.*, 2016) In vivo, after tail vein injection of DOX-loaded GNS@PNV in nude mice bearing subcutaneous Bel-7402 human hepatoma xenografts, NIR laser irradiation triggered the rupture of the gold layer and resulted in the release of DOX. A synergistic effect could be observed in mice treated with DOX-loaded GNS@PNV and laser, as suggested by the tumor inhibition rate and survival of the animals (Figure 11.5 b, c).

Fig 11.5 a) Preparation of DOX-loaded polymer vesicles and gold nanoshells. b), c) In vivo efficiency of the thermo-chemothermal therapy in mice with Bel-7402 human hepatoma xenografts. Reprinted from Biomaterials, 78, Wang *et al.*, “Photothermal-chemotherapy of cancer employing drug leakage-free gold nanoshells”, 40-49, Copyright (2016), with permission from Elsevier.

Gold nanocages

Gold nanocages are very promising nanostructures for combined photothermal-chemotherapy due to their morphological features - hollow interior (allowing for drug loading) and porous walls (which can be easily functionalized) - and tunable LSPR peaks in the NIR region. Qu and co-workers reported an example of a pH- and NIR light-responsive smart nanoplatform for thermo-chemotherapy. (Shi *et al.*, 2012) DOX was loaded inside Au nanocages and then the pores were capped with pH-responsive calcium phosphate-coated iron oxide NPs to protect the cargo. After cell internalization, the acidic pH of subcellular compartments such as endosomes and lysosomes triggered the release of DOX, which was further potentiated by NIR laser irradiation.

The recent work of Yu *et al.* illustrates the potential of gold nanomaterials for the development of multimodal therapeutic solutions for multidrug resistance in cancer. (Yu *et al.*, 2017) The authors developed a system for simultaneous chemo/photothermal/photodynamic therapy, based on the use of Au nanocages loaded with a chemotherapeutic drug (DOX), a photosensitizer (indocyanine green, IG) and a phase-change material (PCM). PCM acted as a “gate keeper” and enabled a controlled release of the cargo molecules upon heating, as the molecule chosen (1-tetradecanol) has a melting temperature of 39°C. The nanocages were surface-functionalized with

biotin for active targeting purposes (DOX/ICG/biotin-PEG-AuNC-PCM, Figure 11.6). Interestingly, *in vitro* using resistant breast cancer cells MCF-7/ADR, DOX release resulted more efficient under NIR irradiation than under global heating to 40°C. Moreover, confocal laser scanning microscopy (CLSM) showed differences in the subcellular localization of DOX (nuclei or lysosomes), depending on the release trigger (NIR irradiation or global heating at 40°C, Figure 11.6 b): under NIR irradiation, the DOX was mainly found inside nuclei, while in the case of cells exposed to global heating DOX accumulated preferentially inside lysosomes. However, further studies are required in order to elucidate the exact mechanism of this preferential subcellular localization of DOX.

Fig. 11.6 a) Schematic of the functional gold nanocage (A) and proposed mechanism for the intracellular release of DOX and ICG (B, C). b) CLSM images of MCF-7/ADR cells incubated with DOX/ICG/biotin-PEG-AuNC-PCM, showing the intracellular localization of DOX. Nuclei were stained in blue with Hoechst 33342, lysosomes in green with Lyso Tracker Green; red signal is from DOX. Reprinted from *Acta Biomaterialia*, 59, Yu *et al.*, “A new NIR-triggered doxorubicin and photosensitizer indocyanine green co-delivery system for enhanced multidrug resistant cancer treatment through simultaneous chemo/photothermal/photodynamic therapy”, 170-180, Copyright (2017), with permission from Elsevier.

11.4 Carbon-based nanomaterials for cancer thermo-chemotherapy

In recent years, besides magnetic and gold nanoparticles, carbon-based nanomaterials have been widely explored for biomedical applications. The raw material of these nanostructures is the graphite, which has a layered and planar macromolecular arrangement based on a honeycomb lattice of sp^2 carbon atoms (Figure 11.7). This particular structure represents the starting material for the synthesis of different types of carbon-based nanomaterials due to the interesting electrical, optical, thermal and mechanical properties that it confers. Among all of them, carbon nanotubes (CNTs) and graphene are the most commonly studied for cancer therapy applications. (Cha C, Shin S R, Annabi N, Dokmeci M R, 2013)

Figure 11.7 Different types of carbon-based materials. Adapted with permission from (Cha C, Shin S R, Annabi N, Dokmeci M R, and K. A. (2013) 'Carbon-Based Nanomaterials: Multi-Functional Materials for Biomedical Engineering', ACS Nano, 7(4), pp. 2891–2897. doi: 0.1021/nn401196a). Copyright (2013) American Chemical Society.

11.4.1 Photo-chemothermal therapeutic applications of carbon nanotubes

Carbon nanotubes (CNTs) were described for the first time by Iijima *et al.* in 1991 as needle-like structures comprising coaxial tubes of graphitic sheets, ranging in number from 2 up to about 50. (Iijima 1991) Two years later, the synthesis of single shell tubes with well-defined diameters was reported and set the basis for the examination of the properties of these structures. (Iijima & Ichihashi 1993) According to their structure, CNTs can be classified into two groups: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs). SWCNTs consist of a single layer of graphene, while MWCNTs are composed of several graphene sheets concentrically

arranged. Depending on the synthetic method, different types of CNTs with different morphologies and sizes can be obtained. Generally, SWCNTs have a diameter from 0.4 to 3.0 nm, with lengths is on the micrometer scale. In the case of MWCNTs, depending on the number of layers, the diameter can range from 2 to 30 nm, while their length can be of 1 to several micrometers, or even longer. (Eatemadi *et al.*, 2014)

These particular nanostructures have recently emerged as powerful alternatives to magnetic and gold nanoparticles in the field of cancer therapy, due to their singular properties. One of the most remarkable properties of CNTs is their photothermal capacity. CNTs can absorb infrared (IR) radiation with between 700 and 1100 nm, and convert it efficiently into heat. This effect occurs due to the fluctuation of electric field on the CNT, promoting the absorption of electromagnetic energy that subsequently induces a rapid heat release. (Levi-Polyachenko *et al.*, 2009) CNTs also stand out for their hollow structure and their high drug loading capacity. Aromatic drugs (i.e. doxorubicin) can be easily adsorbed by π - π stacking and reach loading values of about 70-80% in weight. (Bao *et al.*, 2016) (Liu *et al.*, 2007) On the other hand, achieving a reproducible synthesis and uniform preparation of CNTs is much more complicated than in the case of magnetic and gold nanoparticles. Thus, they have not yet been tested in human clinical trials. (Bianco, Kostarelos and Prato, 2008; Singh and Torti, 2013; Amenta and Aschberger, 2015)

Cancer therapy based on CNTs can make use of the photothermal capacity of CNTs, resulting in a photothermal therapy (PTT) approach, or exploit the synergy between PTT and chemotherapy. For cancer therapy applications involving CNTs, three important aspects have to be tackled: their hydrophobic nature, the active targeting of the ailing tissues and the heating capacity of the CNTs used. One of the most important limitations of CNTs for their use in biomedical applications is their insolubility in water. Thus, an appropriate functionalization is required to improve their biocompatibility, for which different strategies can be used. (Zhang, Bai and Yan, 2016) Covalent and non-covalent functionalization of CNTs with different types of molecules, like proteins (Shim *et al.*, 2002), DNA (Singh *et al.*, 2005), polymers (Liao and Zhang, 2012) or surfactants (Yu *et al.*, 2007) have been described. Moreover, a good targeting of the tumor tissue is mandatory in order to reach a high efficiency of the treatment. (Bhirde *et al.*, 2009) Regarding the heating capacity of CNTs, depending on the final application (PTT or combination with chemotherapy) the selection of the appropriate CNTs is crucial. This is because the heating capacity depends on several factors as the number of walls in MWCNTs (Ghosh *et al.*, 2009), the dimensions of the tubes (Torti *et al.*, 2007) or the concentration used (Burke *et al.*, 2009). For this reason, for photothermal treatment of cancer MWCNTs are more useful than SWCNTs due to the faster rate on NIR absorption and light-to-heat conversion. However, the localized high temperatures that can be attained with MWCNTs can directly ablate the tumor and have side effects on the surrounding tissue. Therefore, for the combined photothermal-chemotherapy approach in which mild hyperthermia conditions are desired, SWCNTs can exhibit a better behavior.

In spite of the good results obtained with PTT of cancer based on CNTs (Shi Kam *et al.*, 2005; X. Liu *et al.*, 2011; Sobhani, 2017), several studies have demonstrated that the synergy between hyperthermia localized in the tumor and chemotherapy represents an important advance in the efficacy of the treatment. Levi-Polyachenko *et al.* described the use of CNTs in combination with chemotherapy for the treatment of peritoneal dissemination of colorectal cancer. (Levi-Polyachenko *et al.*, 2009) The traditional method of treatment of this metastatic disease consist on the surgical removal of the accessible tumor, followed by a classical hyperthermic chemotherapy with an

intraperitoneal perfusion of chemotherapeutic agents warmed to 42°C for 2 h. The authors proposed the use of CNTs to induce a localized hyperthermia in the tumoral cells. By combining oxaliplatin and mitomycin C (two of the most common chemotherapeutic agents used in colorectal cancer) with MWCNTs and NIR laser, a fast increase of the drug delivery on colorectal cancer cell lines (HCT116 and RKO) was observed. This effect was attributed to the enhanced cell membrane permeability due to the heat generated (up to 42°C in just 10 seconds). As a proof of concept, in vitro cell viability assays proved that when nanotubes were added in the presence of the chemotherapeutic agents and infrared stimulation (1064 nm at 3 W of power) was applied for approximately ten seconds, cell survival was reduced to a level comparable to cells incubated at 42°C for two hours. These results show that cell viability can be reduced to seconds using MWCNTs compared to the two hours of the traditional treatment, and hence open the door to a more effective therapy. Nevertheless, further optimization is needed in terms of specific targeting of the tumoral cells.

A controlled release of the chemotherapeutic inside the body is crucial for increasing the effectiveness of the treatment and reducing its side effects. Besides, it has been demonstrated that a prolonged release of moderate concentrations of drug into the target cells can be more beneficial than the release of a large dose of drug. (Moghimi, Hunter and Murray, 2001) CNTs can be engineered to incorporate a chemotherapeutic drug in their coating; the drug can be released inside the target cell or tissue upon application of an external stimulus. Wang *et al.* described a NIR/pH-responsive drug delivery system based on SWCNTs poly(ethylene imine) (PEI), linked with the tumor targeting peptide NGR (Asn-Gly-Arg) and loaded with doxorubicin (DOX). (L. Wang, Shi, Jia, *et al.*, 2013) The release kinetics of DOX from the SWNT-PEI/DOX/NGR delivery system presented a sustained release profile over eight days with a clear dependence on the pH of the medium. Low pH values increase the protonation of NH₂ groups of DOX and its solubility, thus reducing the interactions between DOX and SWCNTs (similarly to some examples described in the previous section for magnetic NPs). As tumoral cells have lower pH values than normal tissues (Kato *et al.*, 2013) the DOX release is promoted. In addition, under 808 nm NIR laser irradiation, the release of the chemotherapeutic drug increased significantly. This kind of kinetics release represents an advantage in terms of having a constant drug concentration in the target cells and a NIR-triggered additional drug release. In vivo, intravenous administration of the system in MCF-7 tumor-bearing mice models confirmed its antitumoral effect resulting from the NIR/pH-responsive drug release, the NGR tumor targeting and the hyperthermia effect of the SWCNTs (Figure 11.8).

Figure 11.8 a) Synthesis and functionalization of SWNT-PEI/DOX/NGR. b) Average tumor size in MCF-7 tumor bearing mice models under treatment with (b) or without (c) 808 nm NIR laser irradiation in vivo. Adapted from Pharmaceutical Research, “NIR-/pH-Responsive drug delivery of functionalized single-walled carbon nanotubes for potential application in cancer chemo-photothermal therapy”, 30, 2013, pp. 2757–2771, Wang, L. *et al.*, (Copyright © 2013, Springer Science+Business Media New York), with permission of Springer.

Hou *et al.* reported a multifunctional system for drug delivery and magnetic resonance imaging (MRI) based on SWCNTs that presents a redox-sensitive behavior. (Hou *et al.*, 2016) Tumor tissues are highly hypoxic and present a high redox potential due to the presence of intracellular reducing molecules, especially glutathione (GSH). In order to exploit this environment for a selective release of drug, SWCNTs were functionalized with hyaluronic acid (HA) and DOX covalently attached by a disulphide bond. Finally, gadolinium (Gd) was adsorbed on the SWCNTs as MRI contrast agent. Inside tumor cells, the disulphide bond is prone to cleavage due to the thiol-disulfide exchange reaction with the GSH, thus providing a fast release of the drug. In addition, HA enhances the targeting and uptake by cancer cells with overexpression of CD44 receptors. In vitro experiments in MCF-7 breast cancer cells demonstrated that the highest cytotoxicity was reached when Gd/SWCNTs-HA-ss-DOX were combined with NIR irradiation. In particular, there was 80% cell growth inhibition induced by NIR at the equivalent DOX concentration of 5 g/mL at 48 hours, in comparison with 62% cell growth inhibition without NIR irradiation. These results were consistent with the ones observed in S180 sarcoma-bearing mice. Tumor size in mice intravenously injected with Gd/SWCNTs-HA-ss-DOX and laser-irradiated (808 nm, 2 W/cm³ for 1 min) decreased significantly in comparison with the injection of the equal concentration of free DOX or a non-redox responsive system (Gd/SWCNTs-HA-DOX). All these results confirmed the excellent chemo-photothermal anticancer therapeutic efficacy of the redox-sensitive nanoplatform.

The combination of CNTs with other kinds of nanomaterials has been also explored for the controlled release of chemotherapeutic agents in combination with NIR

irradiation. Recently, Liu *et al.* developed a system of SWCNTs coated with mesoporous silica (MS) and polyethylene glycol (PEG). (Liu *et al.*, 2015) Doxorubicin was loaded on the mesoporous silica shell and its release was observed to be more sensitive to heat than in a system where the drug interacts through π - π -stacking with the nanotube (SWCNT@PEG/DOX). This represents an advantage because photothermal heating could instantly trigger DOX release due to the weaker DOX-system interactions. In vivo experiments were carried out in 4T1 tumor-bearing mice to assess the synergy between the photothermal therapy and chemotherapy. Tumor growth in mice treated with SWNT@MS-PEG/Dox was effectively inhibited after NIR laser irradiation. This synergy can be explained because the photothermal heating induced by NIR irradiation rapidly increased the temperature to $\sim 48^{\circ}\text{C}$, enhancing the cell membrane permeability and the internalization of the system. Even more, the NIR-triggered drug release further enhanced the cell killing efficiency. Another example of hybrid nanomaterial for cancer thermo-chemotherapy consists of SWCNT-based injectable thermo-sensitive hydrogels (SWNT-GEL). (Zhou *et al.*, 2015) The gel was based on the use of a thermo-sensitive polymer, poly(*n*-isopropyl acrylamide) (pNIPAM), which has a solution to gelation transition at 33°C , thus having a gel form at body temperature. In vitro, no cytotoxicity on BGC-823 (gastric cancer) cells could be attributed to SWCNT-GEL, but a reduction of 30% in the cell viability was observed after NIR irradiation, confirming the hyperthermia effect induced by SWCNTs. Intratumoral administration of DOX/SWCNT-GEL to mice bearing xenograft gastric tumors, together with NIR irradiation, showed a higher inhibition of tumor growth in comparison with the mice treated with only free DOX or SWCNTs-GEL, confirming the synergistic effect of chemotherapy and PTT. Histopathology studies on the four main organs (heart, liver, kidney and spleen) revealed no toxicity or tissue damage, thus proving the biocompatibility of the platform.

11.4.2 Graphene-based nanomaterials for cancer thermo-chemotherapy

Graphene is a monocrystalline graphitic film that was described for the first time by Novoselov *et al.* in 2004. (K. S. Novoselov, A. K. Geim, S. V. Morozov, D. Jiang, Y. Zhang, S. V. Dubonos, 2004) As it has been described previously, the rolling up of these films generates tube structures with very powerful applications in cancer therapy. However, the planar configuration also presents very promising properties in itself. In particular, graphene-based materials are excellent NIR light absorbers and possess a highly specific surface area to which various therapeutic molecules can be conjugated or absorbed, thus facilitating the synergistic effects of combined PTT and chemotherapy.

Although as in the case of CNTs the high efficacy of PTT alone has been proved for graphene, (Robinson *et al.*, 2011; Li *et al.*, 2012; Jung *et al.*, 2014), in this section we will focus on its use for combined PTT and chemotherapy. As for CNTs, the particular arrangement of the carbon atoms on the graphene sheets favors the physical adsorption of chemotherapeutic agents by π - π stacking. This arrangement, together with the ultrahigh surface area, makes graphene the best drug carrier in terms of loading capacity (values of 200% loading in weight can be obtained). (Wu, An and Hulme, 2015) In addition, graphene oxide (GO), obtained by oxidative exfoliation of graphite, can behave as a pH-sensitive carrier. Under acidic conditions, the H bonds between the hydroxyl ($-\text{OH}$) of the GO and the carboxyl ($-\text{COOH}$) and amino ($-\text{NH}_2$) of DOX change, triggering the release of the drug (amine groups of DOX are protonated and

therefore cannot participate in hydrogen bonding). (Yang *et al.*, 2008) This particular behaviour implies an extra advantage in terms of selective release inside tumor tissues due to their more acidic extra and intracellular environments. For the use of graphene in chemo-photothermal cancer therapy, focus must be on the appropriate functionalization of graphene to improve its biocompatibility, blood retention time and selective targeting of tumoral cells, much along the aspects discussed above for carbon nanotubes.

Zhang *et al.* developed a doxorubicin-loaded PEGylated nanographene oxide (NGO-PEG-DOX) system of 100 nm in lateral width for cancer photo-chemotherapy. (Zhang *et al.*, 2011) The system, with a DOX loading of 142.5 wt%, was easily internalized in a murine mammary tumor line (EMT6), in just 2 h. In vitro cytotoxicity studies after irradiation with NIR light (808 nm, 2 W/cm², 3 min) at a DOX concentration of 10 µg/mL showed that the inhibition rate of NGO-PEG-DOX was significantly larger (82.1%) in comparison with free DOX (57.1%) and NGO-PEG alone (45.5%), indicating an enhanced cell-killing effect. For in vivo studies, EMT6 tumor-bearing mice were divided into 4 groups (Figure 11.9). The administration was done intravenously through tail veins and then the tumor region of NGO-PEG group and NGO-PEG-DOX group were irradiated with NIR light (2 W/cm², 5 min), 24 h post-injection. A complete elimination of the tumor was only achieved in the case of NGO-PEG-DOX group in four of the five mice treated, proving that chemo-photothermal treatment based on NGO-PEG-DOX was superior to chemotherapy or photothermal treatment alone.

Figure 11.9 (A) Tumor growth curve after different treatments: (1) PBS (200 µL), (2) DOX (10 mg/kg, 200 µL), (3) NGO-PEG (200 µL, 7 mg/kg), (4) NGO-PEG-DOX (200 µL, 10 mg/kg, in terms of DOX). (B) Evolution of EMT6 tumor-bearing mice during 30 days after treatment. Mice treated with NGO-PEG-DOX + NIR irradiation eliminate completely the tumor. (A) Tumor growth curve after different treatments. (B) Evolution of EMT6 tumor-bearing mice during 30 days after treatment. Mice treated with NGO-PEG-DOX + NIR irradiation eliminate completely the tumor. Adapted from Biomaterials, 32, Zhang, W. et al., “Synergistic effect of chemo-photothermal therapy using PEGylated graphene oxide”, pp. 8555–8561., Copyright (2011), with permission from Elsevier.

The previous example demonstrates that PEGylated graphene materials can be easily uptaken by cells, accumulate in tumors via passive targeting and furthermore, exhibit lower systemic toxicity than the use of free therapeutic agents. However, strategies based on passive targeting usually require large dosages of nanomaterial and chemotherapeutic to achieve sufficient treatment efficacy. As highlighted throughout this chapter, the use of molecules that promote an active targeting can reduce the dose and enhance the efficacy of tumor growth inhibition. Yang *et al.* reported a targeted

delivery system consisting of PEGylated nanographene oxide (NGO) loaded with epirubicin (EPI) and anti-epidermal growth factor receptor (EGFR) antibodies (C225) (PEG-NGO-C225/EPI) as a powerful agent for combined chemotherapy, photothermal therapy and inhibition of EGFR growth signal. (Yang *et al.*, 2013) EGFR is a receptor tyrosine kinase overexpressed in several solid tumors and plays a key role on proliferation, apoptosis, differentiation and migration. (Scaltriti and Baselga, 2006) Then, its downregulation restricts the proliferation cycle and subsequently, induces cell death. In vitro studies on glioma cell lines U87 (overexpressing EGFR) proved that the concentration needed to induce 50% inhibition of cellular growth (IC_{50}) of PEG-NGO-C225/EPI was significantly lower ($9.7 \mu\text{g/mL}$) than the one of free EPI ($15.1 \mu\text{g/mL}$) and of PEG-NGO/EPI ($13.2 \mu\text{g/mL}$), proving the contribution of the C225 to the cell death. Moreover, after the combination with NIR irradiation (2 W/cm^2 , 2 min), chemotherapy and growth signal inhibition resulted in an IC_{50} value of only $2.6 \mu\text{g/mL}$ for PEG-NGO-C225/EPI. This additional effect induced by the high photothermal capacity of NGO was attributed to the breakage of DNA double-strands of tumor cells due to the high temperatures reached ($\sim 70^\circ\text{C}$). The targeting properties of the system were evaluated in U87 tumor-bearing mice after jugular vein injection. An approximately six-fold higher accumulation of PEG-NGO-C225/EPI was obtained compared to PEG-NGO/EPI. In addition, the complete tumor elimination was achieved only for mice that were laser-irradiated (2 min, 2 W/cm^2) after 3 days of a single dose of 6 mg/kg PEG-NGO-C225/EPI containing 3 mg/kg EPI injection.

The combination of graphene with other nanomaterials such as gold, magnetic or polymeric nanoparticles can do the final hybrid system of improved or emerging properties. In this regard, Xu *et al.* reported the use of AuNRs coated with a shell of nanographene oxide (NGO) to improve their properties as chemophotothermal cancer therapy agents. (Xu *et al.*, 2013) AuNRs were encapsulated in NGO through electrostatic self-assembly. Adipic acid dihydrazide (ADH)-modified hyaluronic acid (HA) was covalently bonded to the carboxylic groups of the GO and finally DOX was loaded by π - π -stacking and hydrophobic interactions. This complex (NGOHA-AuNRs-DOX), apart from improving the biocompatibility and hydrophilicity of single AuNRs, enhanced their photothermal effect and increased the targeted towards hepatoma Huh-7 cells through the HA-C44 receptors recognition. In vitro experiments showed a pH-sensitive, NIR-triggered drug release and revealed a 1.5-fold and 4-fold increase of cell death rates compared to single chemotherapy and photothermal therapy, respectively. Song *et al.* developed a hybrid reduced graphene oxide (rGO)-loaded ultrasmall plasmonic gold nanorod vesicle (rGO-AuNRVe) with improved photoacoustic performance and photothermal effects. (Song *et al.*, 2015) Doxorubicin loaded in the vesicles and adsorbed on the surface of the rGO was efficiently released over an 80% of the initial load, promoted by the acidic environment of U87MG cancer cell and NIR irradiation at 808 nm (0.25 W/cm^2). Furthermore, in vivo studies proved a delay of tumor growth in the rGO-AuNRVe-DOX-treated mice after laser irradiation under the same conditions. These results confirmed the synergistic therapeutic effect of the in vivo integrated chemo-photothermal therapy. Wang *et al.* developed a multifunctional drug delivery system based on mesoporous silica-coated graphene oxide. The system was coated with PEG for biostability and interleukin 13 peptide (IL-13) moieties were linked as targeting peptide (IP) for glioma cell targeting. Finally, doxorubicin was loaded in the system by pore absorption and π - π stacking (Figure 11.10). (Y. Wang *et al.*, 2013) The enhanced dispersity of the targeting peptide-modified mesoporous silica-coated graphene nanosheet (GSPI) system improved its NIR absorbance in comparison with the pure graphene (G) or silica coated one (GS). An increase of pH-responsive release of DOX was achieved by NIR heat stimulation due to the dissociation of the interactions between DOX and GSPI. At pH 5.0, the cumulative release of DOX (17%) with NIR irradiation was about 3-fold greater than that without NIR irradiation (5.9%).

This means that the photothermal effect of GSPI could significantly increase the sensitivity of chemotherapy by breaking the strong interactions between graphene-DOX and silica. In addition, in vitro results proved that the targeting peptides promoted the uptake of the system by glioma cells (U251) and GSPI with NIR irradiation mediated the highest rate of cell death compared to a single treatment of GSPI (chemotherapy) or GSPI with NIR irradiation (photothermal therapy).

Figure 11.10 Design of GSPI system for photo-chemotherapy glioma targeting. Reprinted with permission from (Wang, Y. et al. (2013) “Multifunctional mesoporous silica-coated graphene nanosheet used for chemo-photothermal synergistic targeted therapy of glioma”, *Journal of the American Chemical Society*, 135(12), pp. 4799–4804. doi: 10.1021/ja312221g). Copyright (2013) American Chemical Society.

11.5 Conclusions and perspectives

Nanotechnology holds a great promise for combined thermo-chemotherapy of cancer, as highlighted in this chapter with several examples involving magnetic, gold and carbon-based nanomaterials. Perhaps the most important aspect is the possibility of constructing multifunctional nanoplateforms combining targeting, therapeutic and diagnostic/imaging capabilities in a single “magic nanobullet”. Moreover, the use of hybrid nanomaterials can capitalize on the unique properties of each individual component. (Liao *et al.*, 2015; F. Wang *et al.*, 2016; Shen *et al.*, 2016; Chen *et al.*, 2017; Yanping Liu *et al.*, 2017; Wei *et al.*, 2017) Another aspect worth mentioning is the fact that although this chapter focused on chemotherapy, nanomaterial-induced hyperthermia can be also used for gene therapy (L. Wang, Shi, Zhang, *et al.*, 2013; Yin *et al.*, 2015) or immunotherapy. (C. Wang *et al.*, 2014; Yang Liu *et al.*, 2017) Although to the best of our knowledge, no clinical trial has been conducted so far with nanomaterials using the combined thermo-chemotherapy approach, the previous FDA approval of nanoformulations for cancer therapy is very promising. (Li, Liu and Chen, 2017; Riley and Day, 2017) Nevertheless, important aspects must be taken into account in order to ease the transition from the bench to the bedside. For instance, carbon-based materials require a much more in-depth evaluation of their biodistribution, metabolism and clearance from blood circulation, aspects that are not well understood yet, resulting in the publication of contradictory results. (Johnston *et al.*, 2010)(Nezakati, Cousins and Seifalian, 2014) Another bottleneck in the clinical translation of nanomaterials arises from difficulties associated with their synthesis, in terms of scale up and reproducible and uniform preparations. Finally, another important challenge regarding the use of

magnetic and optical hyperthermia is related to the uniformization of the conditions of application of the alternating magnetic field (frequency, magnetic field amplitude, time) or laser irradiation (time and laser potency). This aspect is of paramount importance in order to enable a direct comparison between experiments carried out by different research groups worldwide.

Nevertheless, through the joined efforts of chemists, physicists, material scientists, biologists and medical doctors, we expect that the field of thermo-chemotherapy will witness significant advances in the coming decades.

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