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Abstract: The utilization of graphene-based nanomaterials combined with magnetic nanoparticles offers key benefits in the modern biomedicine. In this minireview, we focus on the most recent advances in hybrids of magnetic graphene derivatives for biomedical applications. We initially analyze the several methodologies employed for the preparation of graphene-based composites with magnetic nanoparticles, more specifically the kind of linkage between the two components. In the last section, we focus on the biomedical applications where these magnetic-graphene hybrids are essential and pay special attention on how the addition of graphene improves the resulting devices in magnetic resonance imaging, controlled drug delivery, magnetic photothermal therapy and cellular separation and isolation. Finally, we highlight the use of these magnetic hybrids as multifunctional material that will lead to a next generation of theranostics.

Keywords: Graphene, graphene derivatives, magnetic nanoparticles, biomedical application, magnetic resonance imaging, drug delivery, photothermal therapy, cellular separation.

1. INTRODUCTION

The recent discovery of grapheme [1] has produced broad research attention to explore the possibilities of this new material in biomedical applications, mainly due to the characteristic structure and the extraordinary set of physicochemical properties [2]. Graphene is a single atomic layer of sp^2 carbon atoms arranged in a honeycomb lattice of nanometer dimensions. Besides, various derivatives of graphene can also be defined, in relation to the number of layers, layer dimensions and amount of oxygen present in the graphene structure [3]. In this way, in addition to graphene, other graphene-based materials (GBMs) of high importance in biomedical applications appear: graphene oxide (GO), reduced graphene oxide (rGO) and graphene quantum dots (GQDs). GO material is considered a graphene derivative with large amount of oxygen-containing groups, mainly epoxy, carbonyl and hydroxyl groups, while rGO is the reduced form of GO [4]. On the other hand, GQDs consist of quasi-spherical graphene nanoparticles with layer dimensions of less than 10 nanometers and showing a graphene quantum effect [5].

Magnetic nanoparticles (MNPs) have been used in biomedical applications such as biosensing, drug delivery, hyperthermia, magnetic resonance imaging (MRI) and cellular capture [6]. Iron oxide nanoparticles (IONPs) are among the most used nanoparticles for magnetic applications [7]. Depending on their size, IONPs can be divided in: i) superparamagnetic (SPION), when sizes are larger than 50nm; ii) ultrasmall (USPION), with sizes below 50 nm; and iii) micron-sized (MPION), which are almost macroscopic with sizes above 1 μm. However, MNPs present several limitations: for instance, they tend to aggregate and precipitate inside the biological vessels, thus reducing their stability, biocompatibility and efficiency. The combination of MNPs with carbon-based nanostructures has recently attracted huge interest in biomedicine because the resulting hybrids allow to overcome the

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MNP limitations and take advantage of the intrinsic properties of both materials [8]. The controlled growth of these IONPs on any of the forms of graphene surfaces (G, GO, rGO and GQD) opens the door to a new set of materials with enhanced efficiency in the common bioapplications of MNPs, mainly in the area of diagnosis and the treatment of cancer cells. The large surface area of graphene makes these nanoparticles effective drug carriers [9], while the magnetic properties from the IONPs convert them as contrast agents for MRI [10]. Graphene-based hybrids with MNPs are a research trend that has been increasing in the last few years (Fig. **1**).

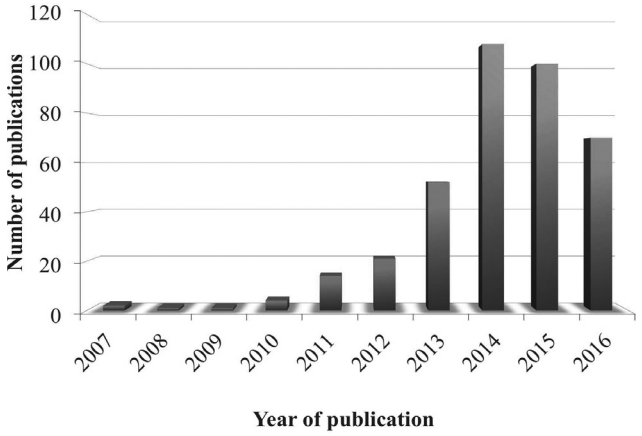

Fig. (1). Number of articles published since 2007 to mid-2016 based on "graphene" and "magnetic nanoparticles", according to Web of Knowledge database.

Several reviews have been published, collecting the application of the different carbon nanomaterials with MNPs in biomedicine [11]. Accordingly, this minireview will focus only on the latest developments in biomedical applications using hybrids based on MNPs with "young" carbon nanoforms, such as GBMs. Particularly, we will highlight the application of GBM-MNP hybrids in MRI, drug delivery, photothermal therapy (PTT) and cellular uptake and isolation. Furthermore, we will pay special attention to multifunctional GBM-MNP hybrids within the above-mentioned applications.

2. PREPARATION OF GBM-MNP HYBRIDS

The preparation of GBM-MNP hybrids involves the modification of graphene materials. There are two main strategies to derivatize GBMs: the covalent and the non-covalent approach [2c,4,12]. In particular, covalent functionalization is the most frequently employed route to modify carbon materials, as this methodology yields products of high stability over time. It is carried out by several methods: the formation of covalent bonds by radical and cycloaddition reactions at sp^2 carbon bonds and the formation of covalent attachment through modification of oxygenated functional groups. On the other hand, non-covalent modifications mainly include hydrophobic and Van der Waals forces, electrostatic interaction, hydrogen bonding, and π-π stacking interaction.

The synthesis of GBM-MNP hybrids can be categorized under two main approaches: *ex situ* and *in situ* methods [11d, 11e]. The former involves a previous synthesis of MNPs and their subsequent attachment on graphene surfaces by either covalent or non covalent modification. This approach may also imply a previous chemical functionalization of both materials [13]. However, the most employed methodology is the *in situ* deposition of MNPs on graphene material. The commonly used MNP precursors are inorganic salts [14] and mineral sources, such as magnetite [15]. Related to the carbon component of hybrids, oxidized graphene materials are excellent platforms for this purpose, as the presence of defects and oxygenated groups favors the growth and attachment of MNPs.

3. BIOMEDICAL APPLICATION OF THE MAGNETIC GRAPHENE

It is well known that any kind of nanoparticles can be delivered to tumoral tissue in two modes: (i) *via* active targeting, where the NPs are functionalized with antibodies and peptides with high affinity to the specific tumor cells receptors; (ii) through passive targeting, according to which positively charged NPs accumulate at tumor sites due to the leaky vasculature and low functional immune system of the area [9]. In any case, the accumulation depends on the nanometer size, the pore diameters of the tumor surfaces, the blood circulation half-life and the degree of tumor vascularization.

3.1. Magnetic Resonance Imaging

The inherent magnetic properties of IONPs make them excellent contrast agents for MRI. However, these nanoparticles tend to aggregate and precipitate inside the body vessels, thus reducing the circulation time in blood and the efficiency as contrast agents for long period imaging. As a solution, graphene-based materials have been employed as a support material to anchor IONPs and enhance the physiological stability of the contrast agent [13a,15a]. Indeed, it was observed that the aggregation of IONPs on GO surface improved

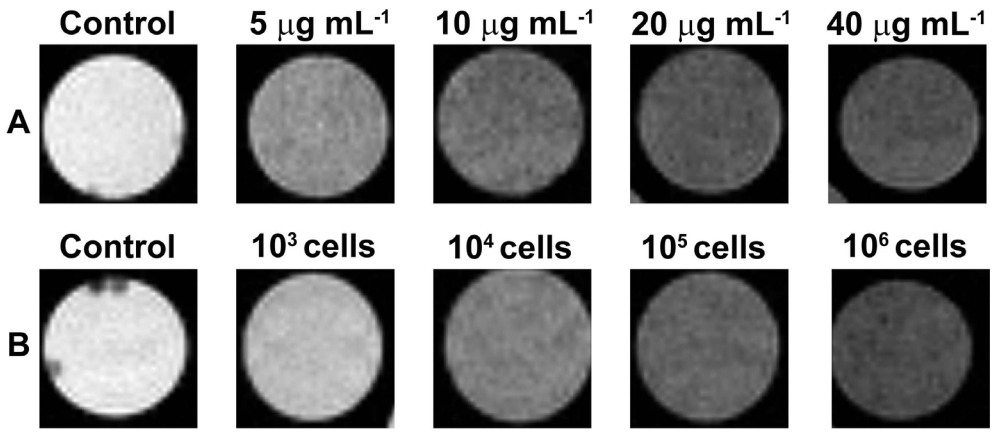

Fig. (2). *T*2 weighted MR images: HeLa cells incubated with the IONPs-GO hybrids at different concentrations (A) and at different cell densities (B) for 24h. Reprinted from Ref. [16], Copyright 2011 American Chemical Society.

significantly the T_2 relaxivity of the system in comparison to the IONPs alone (Fig. **2**).

The first cellular imaging test using aminodextran-coated IONPs on GO sheets was reported in 2011 by Chen *et al* (Fig. **2**) [16]. However, further enhancement of the T_2 relaxivities was desired, in order to apply such hybrids into MRI cellular labeling *in vivo*. In this line, Venkatesha and co-workers demonstrated the importance of the arrangement of the IONPs on the GO surface in GO-IONP hybrids in order to control the transverse proton relaxivity [17]. The authors observed that high amounts of carboxyl groups and a large number of IONPs enhanced the relaxivity value, and concluded that such tendency is due to a synergistic effect between the reduction of GO and the arrangement of IONPs. Chen and co-workers developed a new method to assemble nanoparticles in several morphologies and composition onto GO sheets [18]. They demonstrated that the *in situ* growth of β-FeOOH nanorods onto PEG-GO sheets enhance the transverse relaxivity up to 60 times in comparison to the β-FeOOH-based contrast agents alone. Further studies demonstrated that even at low iron concentrations in the IONPs-PEG-GO nanocomposites, the cellular MRI is always improved [13a].

In summary, there are plenty of studies demonstrating that the presence of graphene-based nanomaterials as contrast agents always enhance the MRI signal, which can be tuned by controlling the aggregation of the MNPs on the graphene framework.

3.2. Controlled-release Drug Delivery Nanocarriers

In the recent years, graphene-IO hybrids have been extensively used for drug release in tumor cells and tissues. The functional groups of GO and RGO have been confirmed to allow higher loadings, since their large surface area allows supporting large amounts of drug [11a, 11d]. However, not only achieving a high drug loading is important for a successful therapeutic effect, but also delivering it to the desired cell or tissue. The superparamagnetic properties of IO are useful here to provide guiding and targeting of the delivery using an external magnetic field and MRI. In summary, graphene-IO hybrids benefit the drug delivery in terms of higher loading and controlled deliveries over graphene or IONPs alone, thus improving the efficiency of the final system.

Doxorubin (DOX) has been regularly used as a chemotherapeutic agent due to its strong cell-killing ability. However, DOX use for cancer treatment is limited, due to its toxic effects. In order to increase the efficacy of this kind of antitumor drugs and reduce its toxicity, it is mandatory to deliver the drugs directly to its target and maintain its concentration during the therapeutic time [19]. Controlled-release nanosystems for drug delivery have become the most promising nanocarriers for therapeutics. Such control is a key feature that can be achieved using switchable gates around the nanocomposite that can be sequentially opened and closed by specific stimuli, such as temperature, pH or light. In this line, Yang and co-workers prepared the first superparamagnetic GO-IONP hybrid with pH-triggered control for controlled targeted drug delivery [20]. In this work, the authors loaded the hybrid system with DOX anticancer agent through physical absorption on the GO surface to test their binding and delivery properties. In addition, due to the inherent optical absorbance of GO in the NIR region, the hybrid was also proposed for photothermal therapy (PTT). In 2013, Balcioglu *et al.* further developed this hybrid by adding AuNPs, resulting in an improvement of the DOX encapsulation [21].

Wang *et al.* incorporated chitosan onto rGO-SPIONs nanosheets to improve their stability, solubility and biocompatibility for cancer chemotherapy and gene therapy [22]. The resulting nanocarrier demonstrated an efficient drug loading capacity, pH-dependent release and good cytotoxicity. DOX was then absorbed on the surface and the resultant composite was encapsulated with a reporter DNA sequence and a green fluorescent protein (GFP) through their interaction with the positively charged chitosan. The delivery of both DOX and DNA was studied *in vitro* and in tumor bearing mice and followed through MRI, and the results demonstrated that the final composite DOX-(chitosan magnetic-G)-GFP-DNA was highly distributed along the tumor site. Furthermore, toxicity studies confirmed that there was no body weight loss of the treated mice.

As well, a carbon nanotube (CNT)-graphene nanosheet (GN)-IONPs hybrid was prepared by loading 5-fluorouracil (5-FU) anticancer drug *via* a green approach and tested as an excellent pH-sensitive therapeutic nanocarrier for anti-cancer drug delivery [23]. The authors observed that the incorporation of the CNT enhanced the transportation of the composite across the cell membrane, as shown by TEM microscopy. Furthermore, they also realized that drug release was higher in the acid environment of the typical cancer cells.

In a recent study, drug nanocarriers based on mesoporous silica-coated magnetic GO were synthesized for anti-cancer drug delivery of DOX [24]. The addition of mesoporous silica increases the surface area, thus drug loading, as well as the cellular uptake. Such carriers were designed with a dendrimer-like structure based on supramolecular poly-pseudorotaxane; such structures are commonly used in targeted drug delivery and act as molecular gates storing the drugs that can be opened by an external stimulus, in this case, a pH change. The resulting system, a part from the pH-sensitivity, has high colloidal stability and positive charge surface, what favors the cellular uptake.

3.3. Magnetic-photothermal Therapy (mPTT)

Although body temperatures above 37ºC are commonly defined fever and associated to an illness, a temperature increase in specific targeted tissues has been observed to have multiple therapeutic benefits in patients with cancer [25]. It has been reported that hyperthermia, a controlled increase of temperatures between 41ºC and 48ºC in localized areas, is clinically relevant for thermal treatments and causes minimal side effects to healthy organs, compared to radiotherapy and chemotherapy [26]. Among the novel tools and techniques, the focus has mainly been addressed to photothermal therapy (PTT), due to its high selectivity and minimal invasiveness. This therapy is based on nanoparticles with photoabsorbing capability to generate heat under NIR irradiation, thus causing thermal ablation of cancer cells. These nanoparticles are normally delivered to tumors before the treatment. Graphene-based structures have become potential agents for PTT due to their high absorbance in the NIR range. Moreover, depositions of IONP on GO produce hybrid nanoparticles that can be magnetically guided to the tumor target and enhance therapy selectivity, as well as improving the light-to-heat conversion [11a]. However, the limited penetration depth of NIR irradiation inside tissues may cause incomplete ablation of large or deep tumors and the use of a high power may damage the neighbor healthy tissues. One solution might be the magnetic localized hyperthermia, which utilizes magnetic nanoparticles directly injected or guided to the tumor area of the patients to induce heat through an external alternating current magnetic field (ACMF). Heat is generated due to hysteresis loss, and induces the apoptosis of tumor cells without damaging nearby healthy tissue [27]. The magnetic properties of these NPs not only permit the magneto-photothermal therapy, but also provide the tool to image and monitor the tumor response to the therapy.

Yang *et al.* developed rGO-IONP-PEG nanocomposites in 2012 and applied them for the first time as agents for magnetic PTT and magnetic guiding *in vivo* [28]. Using the same system, Fu and co-workers treated tumor models through PTT and monitored its response via MRI and analyzed the tumor apparent diffusion coefficient (ADC), concluding that photothermal agents, magnetic guidance and drug-light intervals can all affect the PTT efficiency (Fig. **3**) [29]. Also rGO was combined with superparamagnetic zinc ferrite spinel ($ZnFe_2O_4$) and showed high efficiency as a magneto-PTT agent in low concentrations and minimal cytotoxicity [30].

Hydrogels have been extensively applied in biomedicine due to its hydrated environment and tunable properties similar to the native extracellular matrix. Zhu and co-workers designed a magnetic thermosensitive hydrogel based on GO, IONP and polyethyleneimine carrying DOX [31]. The authors demonstrated not only the successful delivery of DOX, but also an improvement of the therapeutic effect in presence of an ACMF and a reduction of its toxicity.

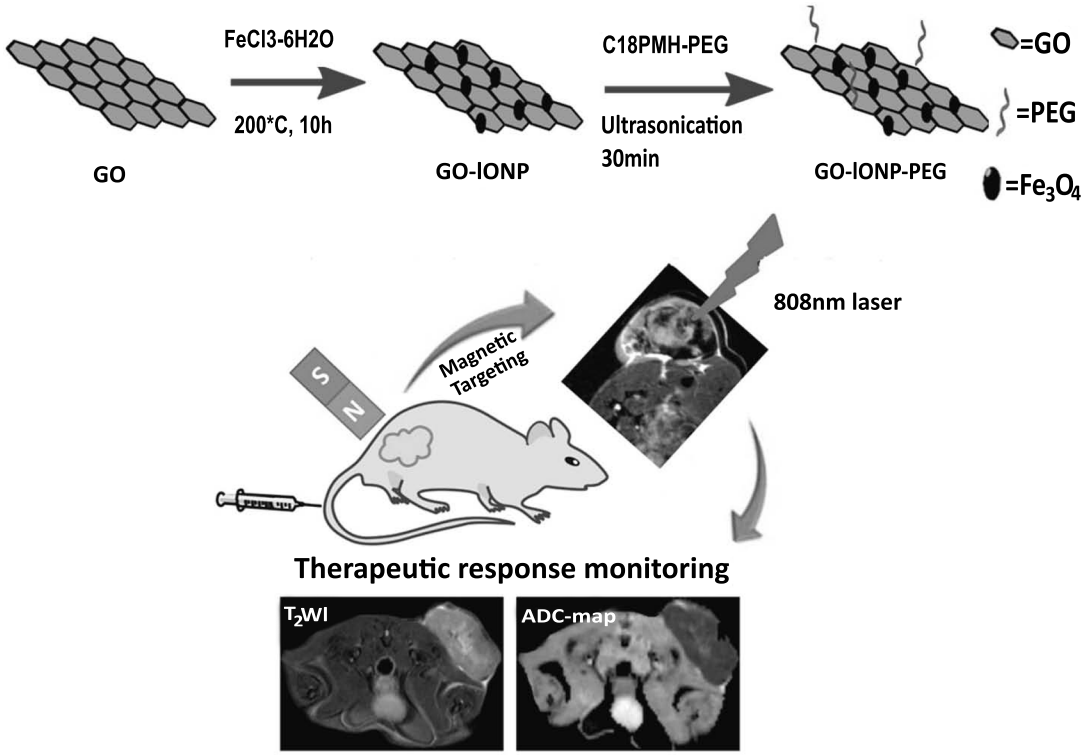

Fig. (3). Preparation of IONP hybrids with PEGylated GO and representation of their PTT application. Reprinted from Ref. [29], Copyright 2016 American Chemical Society.

3.4. Cellular Capture and Isolation

All untreated biological materials, such as the circulating tumor cells (CTCs) and other rare cells, are diamagnetic by nature, thus there exist a chance of separating them selectively from the whole blood using magnetic nanoplatforms and external magnetic currents. In this line, plasmonic-magnetic GO, formed by the attachment of iron and gold nanoparticles, was loaded with anti-GD2 antibody to detect malignant melanoma UACC903 at extremely low concentrations (up to 10 cells per mL) and separate them from whole blood sample using a magnetic bar [32]. In addition, the gold shell allowed the SERS plasmon enhancement, thus improving the detection of the captured cells.

Shi and co-workers have recently developed a novel GOQDs-IONPs hybrid charged with anti-Glypican-3 (GOC3) antibody to capture selectively low-concentrated hepatocellular carcinoma (HCC) tumor cells in an infected blood sample (Fig. **4**). This approach was shown as a very potentially non-invasive tool to detect early stages of liver cancer, with capture efficiencies of about 91% [33]. Furthermore, an aligned Ni-micropillar device decorated with GO and IONPs was developed to control both the capture and the release of cancer cells upon application or removal of a magnetic field [34].

3.5. Multifuctional Applications

Adding IONPs, thus adding magnetic properties, to graphene-based materials, generates new theranostic functionalities to these novel hybrids: not only graphene-IONPs drug nanocarriers can be imaged through MRI, but also, once deposited on tumor sites, can be used as photothermal therapy agents and kill cancer cells very efficiently [35]. Both drug release and PTT are activated through an external stimulus, which is commonly AMF or NIR; however, they have some limitations: NIR has a low penetration inside tissues but a high efficiency in eradicating cancer cells, while AMF has a deeper penetration but narrower efficiency in ablation. Therefore, the combination of both methodologies may provide an outstanding material for drug delivery as well as hyperthermia treatment (Fig. **5**) [36].

In order to exploit the intrinsic properties of graphene-based MNPs, the combination of multifunctional theranostics has gained huge attention in the recent years [13a, 37]. Among the most promising publications, Deng *et al.* synthesized an innovative hybrid "micro-matryoshka" platform comprising GO, IONP and polysaccharides (alginine, chitosan and hyaluronic acid able to bind cancer cells and load drugs through pH control, while dual magnetic and NIR PTT induces

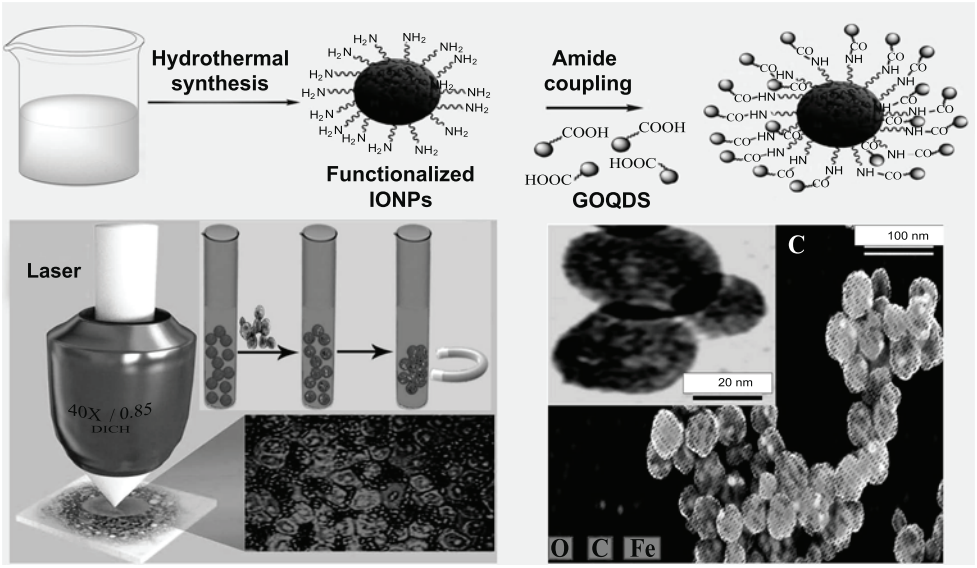

Fig. (4). Schematic representation of the synthesis of GOQDs- IONPs loaded with the antibody GOC3 and their application in the selective Separation of Tumor Cells. Reprinted from Ref. [33], Copyright 2015 American Chemical Society.

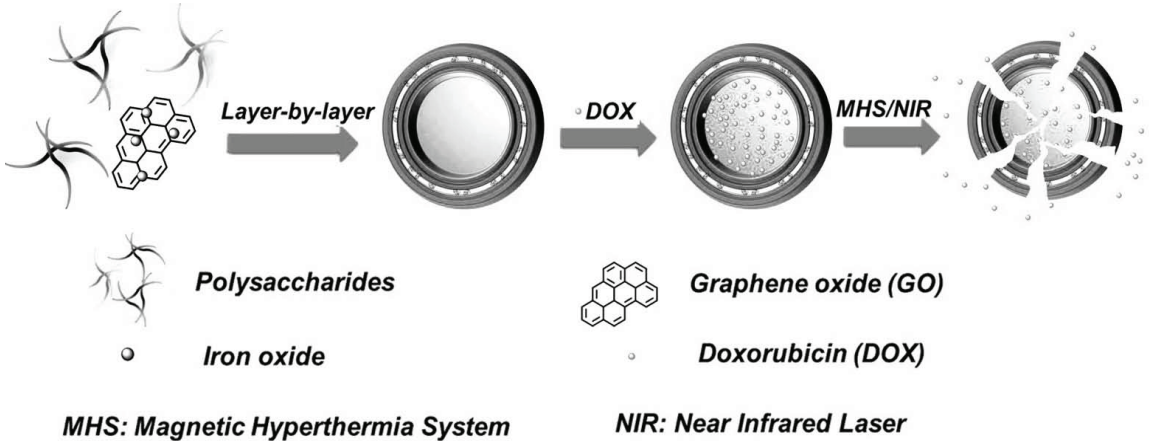

Fig. (5). Schematic illustration of preparation and action mechanism of hybrid capsules synthesized layer-by-layer assembly. Reprinted from Ref. [36], Copyright 2016 American Chemical Society.

hyperthermia ablation of the tumor. The authors performed their successful assays both *in vitro* and *in vivo* and claimed that the whole process was highly controllable [36]. Even more, GO-IONPs hybrids were tested to co-deliver simultaneously several anticancer drugs and, in combination with NIR-induced hyperthermia, were demonstrated to achieve *in vivo* a tumor inhibitory rate of 73.9% [38].

Also iron-cobalt (FeCo) MNPs have been deposited on graphene aiming to saturate the magnetization as an MRI contrast agent. The system was also confirmed to be an excellent DOX delivery system and an exceptional PTT agent [39].

CONCLUSION

The combination of GBMs, carrying remarkable structural and physicochemical properties, with MNP is producing outstanding magnetic hybrids with improved functionalities for nanobiotechnology and biomedicine applications. This fact is clearly reflected in the exponential growth of their publication numbers in the past several years. Although GBM-MNP hybrids are in its "infancy", the preliminary results are encouraging. However, the field is still far from clinical applications, which require addressing some of the remaining challenges. GBMs obviously display many advantages compared with other systems, with the ability to provide efficient MNP loading capacity using very simple preparation procedures. Besides, their intrinsic characteristic allows designing complex multifunctional systems as new direction to produce theranostic agents.

In summary, we have selectively reviewed the ultimate and promising advances in the biomedical applications of GBM-MNP hybrids with special attention in

MRI, drug delivery, PTT and cellular capture and isolation. We believe that such applications will be rapidly enhanced in the forthcoming years.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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