Published in final edited form as:

Nucl Med Biol. 2017 September ; 52: 42–48. doi:10.1016/j.nucmedbio.2017.05.009.

Neutron-Activatable Radionuclide Cancer Therapy Using Graphene Oxide Nanoplatelets

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Abstract

Neutron-activation is a promising method of generating radiotherapeutics with minimal handling of radioactive materials. Graphene oxide nanoplatelets (GONs) were examined as a carrier for neutron-activatable holmium with the purpose of exploiting inherent characteristics for theranostic application. GONs were hypothesized to be an ideal candidate for this application owing to their desirable characteristics such as a rigid structure, high metal loading capacity, low density, heat resistance, and the ability to withstand harsh environments associated with the neutron-activation process. Non-covalently PEGylated GONs (GONs-PEG) offered enhanced dispersibility and biocompatibility, and also exhibited increased holmium loading capacity nearly two-fold greater than GONs. Holmium leaching was investigated over a wide pH range, including conditions that mimic the tumor microenvironment, following neutron irradiation. The *in vitro* cell-based cytotoxicity analysis of GONs-based formulations with non-radioactive holmium confirmed their safety profile within cells. The results demonstrate the potential of GONs as a carrier of neutron-activatable radiotherapeutic agents.

Graphical abstract

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Keywords

Keywords: Neutron-activation; Radionuclide cancer therapy; Graphene oxide; Holmium

1. Introduction

With the advent of the nano-revolution, carbon-based nanomaterials have gained much attention from various fields, and nanomedicine was not the exception [1]. Specifically, carbon nanotubes and fullerenes have been the initial subject of much attention owing mostly to their unique physical and chemical characteristics [2, 3]. Soon after the isolation of graphene by Geim and Novoselov in 2004, this material arguably became the ‘hottest’ carbon-based nanomaterial. Once called “the thinnest material in the universe and the strongest ever measured” [4], graphene has a myriad of attractive qualities: an extremely large surface area, chemical and mechanical strength, and superior thermal and electrical conductivity [5-7].

In the field of nanomedicine, graphene oxide nanoplatelets (GONs) are a preferred choice over graphene as a drug delivery system, because the hydrophobic nature of graphene makes it difficult to disperse in aqueous solvents [8]. Two specific findings dramatically increased the potential of GONs as a drug delivery carrier: they are biocompatible followed by functionalization (e.g., with polyethylene glycol (PEG)), and they exhibit π - π stacking with aromatic-structured chemotherapeutics (e.g., doxorubicin) which offers exceptionally high loading capacity (400 w/w%) [9, 10]. High propensity for metal adsorption is an additional feature that makes GONs attractive as a carrier for therapeutic radionuclides. In one study, graphene oxide was reported to adsorb a 10-fold higher amount of copper ion compared to activated carbon, which has been a heavily investigated material for adsorbents by virtue of large surface area [11]. While there have been a number of published studies assessing the merits of GONs-based delivery of small molecules (e.g., SN38, camptothecin analog, paclitaxel and cisplatin) and large molecules (e.g., vaccines, proteins and genes), publications detailing their utility for delivery of radionuclides are scarce [12-17].

The conventional methods of preparing radiotherapeutics begin with a radioactive isotope which is then conjugated or loaded onto a carrier (e.g. liposomes, polymeric micelles, peptides, or proteins) [18-21]. This method requires the researcher to deal with highly radioactive materials from the very start of the formulation preparation process. Neutron activation – a process by which stable isotopes are converted into radioactive isotopes through the capture of an additional neutron – is a promising approach to minimize the direct handling of radioactivity. Moreover, this strategy is able to offer the additional benefits of minimal use of excipients being able to control the amount of radioactivity produced by varying the neutron flux or irradiation time. In spite of these advantages, the neutron-activation process has not been widely utilized for the production of radiotherapeutics. This is because a significant amount of heat is generated during the neutron-activation process which may result in the degradation of conventional nanocarriers [22]. To overcome this, Di Pasqua et al. previously explored the application of mesoporous silica nanoparticles (MSNs) as neutron-activatable radionuclide carriers. These carriers were found to be able to

withstand the harsh environment required for neutron-activation [23]. However, the relatively high density of the formulation has limited their utility to intraperitoneal administration.

In the current study, GONs were investigated as a lower density and more durable alternative to MSNs for neutron activation and delivery of radionuclides. Among the possible neutron-activatable radionuclide candidates, holmium (Ho) was selected for the current study. Ho possesses desirable characteristics for the proposed application, including its high natural abundance (^{165}Ho can only be activated to ^{166}Ho) and its large neutron capture cross-section (64 barns, cf. 1.3 barns of $^{89}\text{Yttrium}$ where 1 barn = 10^{-24} cm^2) [24-26]. Once stable Ho is activated, ^{166}Ho decays by emission of high energy β - particles ($E_{\text{max}} = 1.84 \text{ MeV}$, 8.7 mm maximum tissue penetration) and low energy γ -photons (81 keV, 6.7% photon yield) which allow for its theranostic application via SPECT/CT imaging (Fig. 1) [27]. The relatively short half-life (26.8 h) reduces the concern of radioactive retention in the human body for extended periods of time. Previous work in our lab involved the synthesis of holmium oxide in the pores of mesoporous carbon nanoparticles (MCNs) as a potential neutron-activated radiotherapeutic [28]. However, incorporation of Ho into the MCNs was a multi-step process that required several steps to achieve complete prevention of Ho leaching. To overcome this limitation, GONs were investigated as a carbon-based carrier for radiotherapeutics that could be loaded in a shorter period of time as well as to achieve better tumor penetration. The successful development of GONs as a neutron-activatable Ho matrix is projected to be utilized for radionuclide therapy which can potentially target multiple targets.

2. Materials and methods

2.1 Chemicals

Graphite flakes, hydrogen peroxide (30%, USP testing grade) and holmium (III) acetate monohydrate (99.99%) were acquired from Sigma-Aldrich (St. Louis, MO). Sulfuric acid (ACS grade), sodium nitrate (analytical grade), sodium hydroxide (ACS grade), nitric acid (trace metal grade) and potassium permanganate (reagent grade) were obtained from Fisher Chemical (Fair Lawn, NJ). 1,2-distearoyl-*sn*-glycero-3-phospho-ethanolamine-N-[methoxy (polyethylene glycol)-3000] (DSPE-PEG) was purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama).

2.2 Synthesis of GONs

GONs were synthesized based on a modification of the graphite flake oxidation and ultrasonication method established by Tour et al. [29]. Briefly, graphite flakes were sieved through a U.S. standard testing sieve (300 μm). A 9:1 mixture of H_2SO_4 and H_3PO_4 (400 mL) in addition to 18 g of KMnO_4 were added to 3 g of sieved graphite flakes and stirred for 12 h at 50 $^\circ\text{C}$. When the reaction had cooled to room temperature (taking on a purple color), 400 mL of Milli-Q water and 3 mL of 30% H_2O_2 were added. The mixture was sieved again (300 μm) and filtered through the polyester fiber. The resultant yellowish filtrate was centrifuged at 2400 x g for 2 h. The filtrate was washed with Milli-Q water, 30% HCl and ethanol (2 times) to remove unreacted precursors, and the washed product was vacuum-dried overnight at room temperature. Graphene oxide was further exfoliated and reduced to nano-

size GONs by 2 h of sonication with probe sonicator (Fisher Scientific Model 505 Sonic Dismembrator; power: 500 watts, operating frequency: 20 kHz and amplitude: 25%). For additional purification, GONs were centrifuged at 1800 x g for 5 minutes and the supernatant was dialyzed for 3 days before their use (MWCO: 12 KDa).

2.3 Non-covalent PEGylation on GONs

In order to enhance colloidal stability and biocompatibility, GONs were PEGylated by a non-covalent method. Since DSPE-PEG was stored in chloroform, the solvent was evaporated and DSPE-PEG was re-suspended in Milli-Q water. Ten mL of GONs (0.5 mg/mL), suspended in Milli-Q water, were mixed with 5 mL of DSPE-PEG (2.5 mg/mL) and sonicated for 10 minutes. The unreacted precursor was removed by centrifugal filtration (3 times, MWCO: 50 KDa).

2.4 Physicochemical characterization of GONs

Fourier transform infrared spectroscopy (FTIR; Thermo Nicolet 380) was used to detect representative functional groups of graphite and GONs. The conversion of graphite to GONs was confirmed by X-ray powder diffraction (XRD; Rigaku Multiflex X-ray diffractometer with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$)). The polydispersity index (PDI) and zeta potential of the GONs were measured using a Malvern Zetasizer Nano ZS. The morphology and thickness of GONs were observed by atomic force microscopy (AFM; Asylum Research MFP3D Atomic Force Microscope), and size distribution was calculated from AFM images by ImageJ.

2.5 Holmium loading on GONs

Holmium acetate ($\text{Ho}(\text{Ac})_3$) was added to either GONs or non-covalently PEGylated GONs (GONs-PEG). Three different loading methods - simple vertexing, short sonication (1-5 minutes), and stirring for 24 h - were compared. To ensure complete loading of Ho on the GON preparations, subsequent experiments were conducted using the short sonication method (5 minutes). To estimate the maximum loading of Ho on the GON preparations, varying amounts of Ho were mixed with the GONs and unbound Ho was removed by centrifugal filtration (MWCO: 50 KDa). The experiment was conducted with ^{166}Ho and the amount was quantified by a γ -counter (PerkinElmer 2470 Wizard γ -counter). The Ho loading capacity was also verified by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500cx with Agilent 1260 Infinity Bioinert LC).

2.6 Holmium desorption from GONs in various pH

^{166}Ho -GONs or ^{166}Ho -GONs-PEG (0.5 mg of GONs/mL) were added to a range of pH solutions which were adjusted by sodium hydroxide and nitric acid titration. Solutions were stirred for 24 h at 100 rpm and then leached ^{166}Ho was separated by centrifugal filtration (MWCO: 100 KDa). ^{166}Ho was quantified using a γ -counter.

2.7 *In vitro* leaching study

Two hundred μL of ^{166}Ho -GONs or ^{166}Ho -GONs-PEG (0.5 mg of GONs/mL) were placed in a dialysis cup (Slide-A-Lyzer MINI dialysis®, 3500 MWCO) which was subsequently

dialyzed against 20 mL of phosphate buffered saline (PBS) at 37 °C and shaken at 100 rpm (Thermo MAXQ 4450). A sample of the dialysate was drawn at pre-determined time points (1, 3, 6, 12, 24, 36 and 48 h) and the leached amount of ^{166}Ho was quantified with a γ -counter.

2.8 Stability of neutron-activated Ho-GONs-PEG

Freeze-dried ^{165}Ho -loaded GONs-PEG were irradiated for 1 h in the PULSTAR Reactor Facility at The North Carolina State University (reactor power = 1 MW; thermal neutron flux = 5.5×10^{12} neutrons cm^{-2} s), and the leached ^{166}Ho was separated by centrifugal filtration (MWCO: 50 KDa) and quantified by γ -counter. The exact amount of Ho in the Ho-GONs-PEG was determined by neutron-activation analysis (NAA). An ORTEC 42% high purity germanium detector system with a Canberra AFT research amplifier, multi-port II analog-to-digital converter and Genie 2000 MCA spectroscopy software was utilized to quantify the amount of ^{166}Ho produced by neutron-activation, which was adopted for back-calculating the amount of ^{165}Ho in the formulation.

2.9 *In vitro* cytotoxicity evaluation

Three non-radioactive GONs-based formulations (GONs, ^{165}Ho -GONs and ^{165}Ho -GONs-PEG) were tested in a human ovarian cancer cell line (A2780) to assess the cytotoxicity of the formulation components. The ovarian cancer cells were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin in 5% CO_2 at 37 °C. When cells became confluent, they were seeded in a 96-well plate (5000 cells/well) and incubated for 24 h. Each well was treated with a pre-determined amount of formulation and cultured for 24 h or 48 h. After removing the formulations and washing with sterile PBS, the media (100 μL /well) and cell counting kit-8 (CCK-8) reagents (10 μL /well, Dojindo Laboratories) were added. Cells were further incubated for 4 h and the absorbance at 450 nm was measured with a plate reader (SpectraMax M5).

3. Results

3.1 Characterization of GONs

To confirm the successful oxidation of graphite, representative functional groups of GONs were analyzed by FTIR (Fig. 2a). The O-H stretching band of the GONs was observed between 3000 cm^{-1} and 3500 cm^{-1} . The C=O stretching region of carboxylic groups and the C-O-C stretching band of the epoxide groups were seen at 1725 cm^{-1} and 1052 cm^{-1} , respectively. A change of XRD pattern from graphite to GONs also supported the successful synthesis (Fig.2b). To exploit passive targeting via the enhanced permeation and retention (EPR) effect, GONs were reduced to nano-size by sonication and they were shown to have an irregular shape with a wide size distribution ($136.7 \pm 85.2\text{ nm}$) (Fig. 3a,c) [30]. The approximate 1.2 nm height of GONs suggested that they were successfully separated from stacked graphite to a single-layered graphene structure, which matches previous reports (Fig. 3e) [29]. The PDI and surface charge measurement by zetasizer exhibited monodispersity (0.230 ± 0.006) and a net negative charge ($-49.8 \pm 3.9\text{ mV}$), primarily from the carboxylate groups of GONs.

3.2 Surface modification of GONs

To obtain a narrow size distribution, solutions were centrifuged to eliminate large-sized GONs, and resulting supernatant portion was used in the PEGylation process. GONs-PEG was shown to have narrower size distribution (62.1 ± 14.0 nm) (Fig. 2b,d). The non-covalent attachment of PEG resulted in an increased cross-section height to ~ 14 nm and a slight improvement in the PDI (0.185 ± 0.003) (Fig 3f).

3.3 Holmium loading on GONs

Three methods were evaluated for loading GONs with $\text{Ho}(\text{Ac})_3$; and no significant difference in loading capacity and efficiency were observed. As shown in Fig. 4, GONs was able to maintain their dispersibility until the weight ratio of GONs: $\text{Ho}(\text{Ac})_3$ reached 1:0.1, at which point above this holmium ratio, the suspensions were observed to aggregate. Based on ICP-MS analysis, the maximum loading capacity of holmium on GONs that could maintain dispersibility was determined to be 4.38 w/w %. When PEGylated, GONs maintained their dispersibility even at GONs: $\text{Ho}(\text{Ac})_3$ ratios of 1:0.2. The maximum holmium loading on GONs-PEG was evaluated by NAA and determined to be 8.38 w/w %.

3.4 Holmium sorption on GONs

The binding of holmium on GONs was evaluated within pH range of 1 to 14. The sorption profiles of GONs and GONs-PEG were not significantly different based on two-way ANOVA analysis. Almost all of the Ho was bound to GONs and GONs-PEG ($> 98\%$) between pH 4-14 (Fig. 5). However, as oxygen-containing functional groups of GONs were protonated in acidic pH, sorption of Ho decreased as the pH. Only $\sim 16\%$ of holmium was bound to GONs and GONs-PEG at pH 1.

3.5 *In vitro* leaching assessment

As expected, there was almost no Ho leaching observed at physiological pH (7.4) until 12 h after incubation (Fig. 6). After 48 h, only 10% of Ho leaching was observed. Unlike GONs, GONs-PEG did not show any Ho leaching at pH 7.4 even after long incubation times. Under slightly acidic conditions (pH 5.5), GONs and GONs-PEG exhibited sustained-release profiles that were not significantly different.

3.6 Stability of neutron-activated GONs

The critical parameter to be assessed for carriers of neutron-activated radiotherapeutics is the leaching of radioactivity following neutron irradiation which may result in degradation of GONs in the relatively harsh reactor environment during the irradiation process. As the ultimate goal is to deliver therapeutic radionuclides to target tissues (e.g., tumors), a minimal amount of leaching of the radionuclide (^{166}Ho in this application) is desirable to prevent off-target accumulation of the released radionuclide. The non-radioactive Ho-loaded GONs and Ho-loaded GONs-PEG were irradiated in a neutron flux for 1 h and washed 3 times with Milli-Q water. Only very small amounts ($<0.1\%$ of the total radioactivity) of ^{166}Ho could be detected in the washings which indicated that leaching of radioactive holmium from these irradiated particles was minimal.

3.7 *In vitro* cytotoxicity evaluation

The viability of a human ovarian cancer cell line (A2780) following exposure to various concentrations of GONs, Ho-loaded GONs (^{165}Ho -GONs), and Ho-loaded GONs-PEG (^{165}Ho -GONs-PEG) was assessed. GONs did not induce any cytotoxicity at concentrations up to 10 $\mu\text{g/mL}$ when incubated for 24 h or 48 h, and the addition of Ho and PEG did not add increase the toxicity of the carriers (Fig. 7).

4. Discussion

In this study, GONs were explored as a neutron-activatable radionuclide carrier. The successful synthesis of GONs was confirmed by FTIR, XRD and AFM. For a GONs-based formulation, surface modification was necessary for passive tumor-targeting effects [31]. Previous studies have demonstrated that GONs undergo irreversible aggregation without proper capping agents [32-34]. The PEG moiety is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) and is frequently employed for enhancing both biocompatibility and dispersibility of nano-carriers in biological systems [35]. Covalent and non-covalent PEGylation strategies were evaluated with the latter ultimately being selected based on ease of the method and the short reaction time (10 minutes of sonication) to exploit the strong association between the phospholipids portion of DSPE-PEG and the aromatic moieties of GONs.

Among the different forms of holmium, $\text{Ho}(\text{Ac})_3$ was selected for incorporation into GONs. $\text{Ho}(\text{Ac})_3$ is a preferred load versus $\text{Ho}(\text{Cl})_3$, as the acetate form does not contain readily activatable nuclei, while radioisotopes of Cl can form upon neutron irradiation of $\text{Ho}(\text{Cl})_3$. Other investigators report the use of cargo loading methods that require incubations for an extended period of time [36-38]. Since GONs are known to possess a large surface area, it was assumed that the Ho loading process would proceed more rapidly with GONs compared to other nanocarriers. There was no significant difference between the three loading methods in terms of loading capacity or efficiency in GONs:Ho (Ac)₃ ratios tested. These results suggest that Ho associated quickly with GONs. Holmium binding on GONs is facilitated mostly through electrostatic interaction with the negatively-charged oxygen-containing functional groups on GONs; electrostatic repulsion results in a stable dispersion between resulting layers [39]. As these negatively charged groups are masked by Ho binding, products started to show aggregation at GONs:Ho(Ac)₃ ratios above 1:0.1. Since PEGylation was able to offer additional dispersibility even after the charge masking, Ho-GONs-PEG did not aggregate with higher Ho ratios, and Ho loading capacity increased from 4.38 (w/w) % to 8.38 (w/w) %.

Ho sorption was investigated over a wide pH range. Some elements such as U(IV), Sr(II), Tc(VII) and Np(V) have weaker and more pH-dependent association with graphene oxides compared to others [40]. Since GONs were not covalently PEGylated in this study, the functional groups of GONs were still intact following PEGylation. Hence, there was no significant difference in Ho sorption between GONs and GONs-PEG. The result of this sorption experiment showed a similar pH-dependent pattern - Ho sorption was nearly complete at pH 4, and significant leaching was seen in acidic conditions - in comparison to other heavy metals (Th(IV), Pu(IV), Am(III), Eu(III)) [40].

Although the sorption experiment demonstrated a strong binding affinity between Ho and GONs, this complex was further evaluated in conditions that mimic the tumor microenvironment. While the pH of human body fluid is well-regulated by homeostasis to be maintained at pH 7.4, the pH of the tumor microenvironment is slightly acidic (pH 5.5 to 7.0) due to glycolytic metabolism, hypoxia, and deficient blood perfusion [41, 42]. Although Ho was shown to bind to GONs quantitatively at pH >4 in the sorption study, holmium was observed to be slowly released from the GONs in the leaching study when the pH of the medium was 5.5 due to a change in equilibrium resulting from the larger volume of dialysate in the leaching study. The faster release rate at pH 5.5 compared to pH 7.4 was likely due to protonation effects at the lower pH. Considering that Ho did not leach from the GONs-PEG at pH 7.4 after a long incubation time, DSPE-PEGylation was considered as a means to offer an additional barrier to holmium loss, but this effect was diminished in acidic conditions (pH 5.5).

One well-known limitation of nanocarrier-based drug delivery system is tumor penetration. A considerable portion of administered nanoparticles remains near blood vessels instead of penetrating into deep tumor region which often causes a sub-therapeutic effect. Cabral et al. showed that smaller nanoparticles (30 nm) were able to penetrate tumor tissue more efficiently than larger nanoparticles (50-100 nm); Wong et al. reported that 10 nm nanoparticles in the tumor microenvironment were capable of reaching deeper tumor sites than 100 nm nanoparticles [43, 44]. Based on these reports, the sustained holmium desorption in a mildly acidic environment may confer improved deep tumor penetration *in vivo*. Lastly, the safe toxicity profile of GONs-based formulations (with non-radioactive holmium) verified that the tumor killing effect would exclusively result from the high energy of β^- particles emitted by ^{166}Ho and not by the toxicity of GONs themselves.

Overall, non-radioactive Ho-loaded GONs-PEG did not show any GONs-related cytotoxicity at concentrations up to 10 $\mu\text{g/mL}$ for 48 h. After 1 h of neutron irradiation which produced radioactive holmium, no leaching of ^{166}Ho was observed when the ^{166}Ho -GONs-PEG were incubated at pH 7.4 for extended periods of time. The results suggest that Ho-loaded nanocarriers will stay intact not only during preparation, but also in the circulation *in vivo* after intravenous administration until they arrive at their target site.

5. Conclusion

Graphene oxide nanoplatelets were evaluated as a neutron-activatable holmium carrier for theranostic application. GONs were synthesized from graphite and subsequently characterized. Through non-covalent PEGylation, their dispersibility and biocompatibility were improved, and the Ho-loading capacity increased nearly two-fold (w/w %). Leaching of radioactive holmium following neutron irradiation of Ho-GONs-PEG was minimal for 48 h which is almost two half-lives of the radionuclide. The safety of this formulation was supported by *in vitro* cytotoxicity assays in a human ovarian cancer cell line. As a platform technology, the neutron activation approach using GONS as the carrier is applicable for several therapeutic radionuclides such as ^{90}Y , ^{153}Sm , ^{177}Lu , ^{188}Re and ^{198}Au . GONs can also be used as a matrix for other therapeutic radionuclides by virtue of their ability to avidly adsorb a variety of metals. The adsorption of high-specific activity radioactive metals

like ^{89}Sr and ^{90}Y to GONs would add an extra handling step, but would avoid the logistical issues of the neutron activation approach such as the availability of a high flux neutron source (e.g., a nuclear reactor). Further experimentation would be required to determine if GONs could be used as carriers for radiotherapeutic anions like ^{32}P -phosphate or ^{131}I -iodide. If the stability of chemotherapeutics entrapped within Ho-GONs-PEG can be confirmed following neutron irradiation, a combination therapy approach (chemoradiotherapy) is anticipated to enhance tumor killing and improve clinical outcomes.

Acknowledgments

The authors are thankful to Dr. A. Kumbhar for guidance in AFM and Dr. J. Prybylski for advice on statistical analysis. We also greatly appreciate S. Lassell at the North Carolina State University Nuclear Reactor Program for assistance with neutron-activation and NAA. This work was performed in part at the Chapel Hill Analytical and Nanofabrication Laboratory, CHANL, a member of the North Carolina Research Triangle Nanotechnology Network, RTNN, which is supported by the National Science Foundation, Grant ECCS-1542015, as part of the National Nanotechnology Coordinated Infrastructure, NNCI. This work was supported by the National Cancer Institute through the Carolina Center of Cancer Nanotechnology Excellence (C-CCNE) Pilot Grant Program (1U54 CA151652-01) and a Dissertation Completion Fellowship from the University of North Carolina-Chapel Hill.

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Fig. 1. Schematic representation of neutron-activatable GONs-based therapy

Fig. 2. Synthesis of GONs confirmed by (a) FTIR and (b) XRD

Fig. 3. Characterization of GONs and GONs-PEG by AFM. AFM images of (a) GONs and (b) GONs-PEG. Size distribution of (c) GONs and (d) GONs-PEG as estimated by ImageJ. Cross-section analysis of (e) GONs and (f) GONs-PEG by AFM

Fig. 4. Evaluation holmium loading on (a) GONs and (b) GONs-PEG (red box indicates the aggregation of the formulation)

Fig. 5. Assessment of holmium sorption on GONs and GONs-PEG in a wide range of pH. Groups were compared by two-way ANOVA ($\alpha = 0.05$)

Fig. 6.

Comparison of in vitro holmium leaching profile between GONs and GONs-PEG at pH 4.5 and 7.4. Groups were compared by two-way ANOVA (** for $p < 0.01$ and **** for $p < 0.0001$). Each time point of same pH groups was analyzed by Bonferroni's multiple comparisons test (δ for adjusted $p < 0.05$ from GONs-PEG (pH 7.4)).

Fig. 7. Viability of A2780 cells following 24 h (a) and 48 h (b) incubation of GONs and non-radioactive holmium-containing GONs and GONs-PEG