Gd@C\textsubscript{82} metallofullerenes for neutron capture therapy—fullerene solubilization by poly(ethylene glycol)-block-poly(2-(N,N-diethylamino)ethyl methacrylate) and resultant efficacy \textit{in vitro}

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Abstract
Poly(ethylene glycol)-block-poly(2-(N,N-diethylamino)ethyl methacrylate) (PEG-\textit{b}-PAMA) was found to solubilize fullerenes such as C\textsubscript{60}, and this technique was applied to metallofullerenes. Gd@C\textsubscript{82} was easily dissolved in water in the presence of PEG-\textit{b}-PAMA without any covalent derivatization, forming a transparent complex about 20–30 nm in diameter. Low cytotoxicity was confirmed \textit{in vitro}. Neutron irradiation of cultured cells (colon-26 adenocarcinoma) with Gd@C\textsubscript{82}-PEG-\textit{b}-PAMA-complexed nanoparticles showed effective cytotoxicity, indicating the effective emission of gamma rays and internal conversion electrons produced from the neutron capture reaction of Gd. This result suggests a potentially valuable approach to gadolinium-based neutron capture therapy.

Keywords: neutron capture therapy, GdNCT, fullerene, Gd@C\textsubscript{82}, PEG-\textit{b}-polyamine, \textit{in vitro}

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1. Introduction
Neutron capture therapy (NCT) is a suitable method for the treatment of intractable tumors such as brain tumors. NCT using \textsuperscript{10}B (BNCT) has demonstrated efficacy in the treatment of tumors [1, 2] and is now established as a frontier radiotherapy. \textsuperscript{10}B compounds based on boronophenylalanine (BPA) and sodium borocaptate (BSH) have undergone clinical trials to verify their efficacy in BNCT because they show low cytotoxicity without neutron irradiation [3–5]. However, since selective accumulation in tumors is insufficient, improvements in targeting characteristics are desirable. We have recently developed boron-containing nanoparticles for passive targeting in tumors, which improves the BNCT efficiency. Another important requirement for high-performance neutron therapy is the monitoring of the biodistribution of compounds, including neutron capture agents. Precise determinations of the capture compound concentration would facilitate minimization of the neutron source power and reduce damage to normal organs by neutron irradiation.

Gadolinium is a promising candidate for monitoring biodistribution by magnetic resonance imaging (MRI)
because of the significant variations in the water relaxation time [6]. Gadolinium has a high neutron capture cross section [7, 8]. Naturally occurring gadolinium has a capture cross section of 48 800 barns for thermal neutrons, higher than that of $^{10}$B (3838 barns) [3]. Both $^{155}$Gd (14.8%) and $^{157}$Gd (15.7%) have high neutron capture cross sections (60 000 and 255 000 barns) [7–9] and are thus extremely useful in gadolinium-based neutron capture therapy (GdNCT) [10–12]. Neutron capture by $^{155}$Gd and $^{157}$Gd produces 8.64 and 7.94 MeV of gamma rays (39–199 and 29–182 keV each, efficiency ~75%) and internal conversion electrons [9, 13, 14]. These energies are high enough for NCT. Note that the energy range from a thermal neutron to an epithermal neutron is only 0.025 eV to 10 keV, which is low enough to suppress direct damage to normal tissues without capture compounds.

Gadolinium ions have a stronger effect on hydrogen-proton spin-lattice relaxation than other ions because of their large number of valence electrons (7 for Gd$^{3+}$) [15]. Owing to this property, gadolinium has traditionally been used in radiocontrast agents in biomedical phases [6]. Since gadolinium ions are very toxic, chelation with specific ligands such as diethyleneetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and hexane-2,4-dione has been used to reduce the toxicity [16–19]. Other radiocontrast agents based on Gd chelates have been proposed to improve the proton relaxivity [20–22] because the relaxation properties of the gadolinium ion depend on its environment [23].

Improvements in GdNCT require the optimization of two factors: the selective accumulation of Gd compounds in tumor tissue and the reduction of gadolinium toxicity. A similar strategy has been employed in magnetic resonance imaging (MRI), wherein the complexation of Gd with a suitable chelating agent has been investigated. Akine et al demonstrated GdNCT using a commercial gadolinium radiocontrast agent (meglumine gadopentetate) [24]. Because this agent does not accumulate well in tumor tissue, meglumine gadopentetate is administered directly to the tumor by subcutaneous injection. For GdNCT, however, the administered dose must be much higher than that of the MRI contrast agent and the resultant toxicity would be unacceptable. To improve blood circulation and tumor site accumulation, several kinds of matrices such as lipids [17, 25], chitosan [26, 27], polymers and ligands have been proposed for GdNCT [28, 29]. Gadolinium-functionalized nanoparticles with improved accumulation properties were developed for high-contrast MRI [30, 31]. These compositions are also useful for GdNCT. However, the toxicity of Gd complexes with longer in vivo retention cannot be ignored. A new strategy must be employed for the use of Gd compounds as neutron trapping agents.

Interest in the chemistry, physics and biology of fullerenes has grown rapidly in recent years. Metallofullerenes containing one or more metal atoms in a fullerene cage are a new class of materials showing unusual electronic and magnetic properties [32]. Gadolinium metallofullerenes are prepared by the arc heating of graphite with gadolinium oxide and are separated from non-caged fullerenes by column chromatography. Since the water solubility of Gd@C$_{82}$ is too low for medical applications, several solubilization strategies have been applied. One technique used to prepare a soluble Gd@C$_{82}$ is hydroxylation of a carbon sheet [33, 34]. Mikawa et al revealed that hydroxyl-modified Gd@C$_{82}$ has a high potential for use as an MRI radiocontrast agent because its proton relaxivity is much higher than that of Gd-DTPA [33]. This high proton relaxivity is attributed to the effect of the highly conductive carbon structure of the fullerene cage on the gadolinium atom.

We originally achieved the solubilization of intact fullerences by using synthetic block copolymers. For example, poly(ethylene glycol)-b-poly(N,N′-(dimethylamino)ethyl methacrylate) (PEG-b-PAMA) solubilizes C$_{60}$ to form extensively mono-dispersed nanoparticles with a diameter of 5 nm [35]. The solubility of C$_{60}$ in PEG-b-PAMA aqueous solution reached 214 mg l$^{-1}$ as evaluated by optical absorption, in contrast to previously reported values such as 3 mg l$^{-1}$ in lecithin, 58 mg l$^{-1}$ in cyclodextrin and 100 mg l$^{-1}$ in fluoroalkyl end-capped water-soluble oligomers [35]. Analogously, we solubilized Gd@C$_{82}$ in aqueous media using PEG-b-PAMA. Because PEG-b-PAMA is located on the surface of the fullerene cluster, the resulting complex is anticipated to have high biocompatibility. The chemical modification of fullerences alters the structure of the carbon sheet and reduces its proton conductivity. PEG-b-PAMA physically interacts with the fullerene to maintain the dispersion of Gd@C$_{82}$, and this better preserves the properties of Gd@C$_{82}$ as compared to chemical functionalization.

In this paper, we demonstrate the solubilization of Gd@C$_{82}$ using PEG-b-PAMA via physical entrapment and apply the Gd@C$_{82}$-PEG-b-PAMA-complexed nanoparticles (GdNPs) to GdNCT. To prepare the GdNPs, the hydrophobic segment (PAMA) of the block copolymer was reacted with Gd@C$_{82}$ using PEG-b-PAMA via physical entrapment and -PEG-b-PAMA-complexed nanoparticles (GdNPs) to GdNCT. To prepare the GdNPs, the hydrophobic segment (PAMA) of the block copolymer was reacted with Gd@C$_{82}$ to form the nanoparticles in dimethylformamide (DMF) upon sonication (figure 1). The solvent was exchanged into a phosphate buffered saline (PBS) buffer for in vitro NCT studies (figure 2).

2. Experimental details

2.1. Materials and reagents

Gd@C$_{82}$ (purity 80–90%) was purchased from MTR Ltd.; it contained Gd@C$_{80}$, Gd$_2$@C$_{78}$ and Gd$_3$@C$_{80}$ (supplementary data, figure S1 available from stacks.iop.org/STAM/12/044607/mmedia). Commercial tetrahydrofuran (THF) and 2-(N,N-dimethylamino)ethyl methacrylate (AMA) were purchased from Kanto Chemical Co. Ltd. 2-Methoxyethanol was purchased from Tokyo Chemical Industry Co. Ltd. Ethylene oxide was purchased from Sumitomo Seika Chemicals Co. Ltd. Each chemical was purified by distillation. Potassium naphthalene was prepared as described previously [36]. DMF (Kanto Chemical Co. Ltd) was used as received. Dialysis membrane with a molecular weight cut off (MWCO) of 12 000–14 000 was purchased from Spectrum Co. Ltd. WST-8 cell proliferation assay kit was purchased from Dojin Chemical Co. Ltd. Dulbecco’s...
modified eagle medium (DMEM), penicillin-streptomycin liquid and fetal bovine serum were purchased from Life Technologies (Invitrogen).

2.2. Equipments

Branson 1200 (Yamato Scientific Co. Ltd), Ultrason VS-30 (Velvo-Clear Co.) and US-107 (SND Co. Ltd) sonicators were used for sonication. Nanoparticle diameters were determined by Zetasizer Nano-ZS (Malvern). A Kubota 3740 centrifuge (Kubota Corp.) and Vivaspin 20 ultrafiltration membranes (MWCO: 5000, GE Healthcare) were used for ultrafiltration. A Multiskan FC microplate reader (Thermo Fisher Scientific KK) was used for western standard time assay (WST-assay).

2.3. Synthesis of PEG-b-PAMA

The procedure was based on anionic polymerization as reported previously [37]. Potassium 2-methoxyethanolate (1 mmol), prepared by the reaction of equimolar 2-methoxyethanol and potassium naphthalene, was reacted with ethylene oxide (120 mmol) to initiate the ring-opening anionic polymerization in THF (35 ml). PEG-b-PAMA was obtained via block copolymerization, which ensued with the addition of AMA (16 mmol) after ethylene oxide consumption was complete. The resultant polymer was precipitated in 2-propanol. The precipitate was dissolved in water (30 ml) and adjusted at pH 5 by adding 1 M HCl. After lyophilization, protonated PEG-b-PAMA was purified by Soxhlet extraction with THF. The product was characterized and its purity determined by gel permeation chromatography and proton nuclear magnetic resonance (supplementary data, figures S2 and S3 available from stacks.iop.org/STAM/12/044607/mmedia). The average molecular weight of each PEG and PAMA segment was 5300 (120 units) and 3200 (20 units), respectively.

2.4. Preparation of GdNPs

Gd@C₈₂ (1 mg) was dissolved in 5 ml DMF and sonicated for 1 h. PEG-b-PAMA (5 mg or 50 mg) was added to the mixture and the sonication continued for 3 h (figure 1). The mixture was transferred to a dialysis tube (MWCO: 12 000–14 000) and dialyzed against 2 litres of water. The external water was changed 4 times at $t = 3, 15, 39$ and $63$ h. The complex was concentrated by ultrafiltration. PBS powder was added to each solution and adjusted to $1 \times$ PBS solution.

2.5. Cytotoxicity test (WST-assay)

Colon-26 adenocarcinoma cells (colon-26 cells) were seeded on a 96-well plate (5000 cells per well) and incubated for 1 day. To each well (containing 80 µl cell culture) we added 20 µl each of GdNPs solution (50, 250 or 500 ppm Gd

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**Figure 1.** Schematic of the preparation process of GdNPs.

**Figure 2.** Illustration of neutron irradiation of culture cells with (right) and without (left) GdNPs.
Figure 3. Illustration and photograph of the sample setup for neutron irradiation (Cyborg 480 System).

2.6. Neutron irradiation

Neutron irradiation was performed at the Japan Research Reactor No. 4 (JRR-4) at the facility of Japan Atomic Energy Agency (JAEA) located in Tokai Village (Ibaraki, Japan). Colon-26 cells were seeded on a 96-well plate (5000 cells per well) and incubated for 1 day. To each well (containing 80 µl cell culture) we added 20 µl each of GdNPs solution (50, 250 or 500 ppm Gd atoms in PBS). The final concentrations of Gd atoms in the wells were 10 ppm (≈63.4 µM Gd, 3.8 mg ml\(^{-1}\) of PEG-b-PAMA), 50 ppm (≈317 µM Gd, 19 mg ml\(^{-1}\) of PEG-b-PAMA) and 100 ppm (≈634 µM Gd, 38 mg ml\(^{-1}\) of PEG-b-PAMA). After incubation for 6 h, each well was washed with a PBS solution and 100 µl of DMEM was added to the well. After 48 h incubation, 10 µl WST-8 reagent was added to each well, the plate was incubated for another hour, and the absorbance of each well was measured with a multi-plate reader at 450 nm.

3. Results and discussion

3.1. Characterization of GdNPs

The proposed application of Gd@C\(_{82}\) to GdNCT requires water solubilization. We have previously confirmed that PEG-b-PAMA block copolymer can be utilized for the solubilization of fullerenes. For example, C\(_{60}\) was solubilized by PEG-b-PAMA forming nanoscale C\(_{60}\) clusters of about 5 nm diameter under suitable conditions [35]. Both the hydrophobic and electrostatic interactions of the PAMA segment contribute to the solubilization of fullerene. The solubilization characteristics of PEG-b-PAMA for Gd@C\(_{82}\) were studied by dynamic light scattering. After the dispersion of the complex in water by dialysis, a transparent, brownish solution was obtained. The size distribution of the resultant GdNPs is shown in figure 4. For a w/w ratio of polymer to Gd@C\(_{82}\) of 5, 25–35 nm diameter particles were observed in addition to a small amount of aggregates (about 200 nm, supplementary data, figure S5). When the relative amount of PEG-b-PAMA was increased (w/w ratio = 50, figure 4), the average particle size and its distribution decreased to 21 and 15–25 nm, respectively. These results indicate that PEG-b-PAMA works as a surfactant to solubilize Gd@C\(_{82}\) in aqueous media via interaction between the hydrophobic fullerene surface and the hydrophobic PAMA segment. The size of the complex could be controlled by changing the polymer/Gd@C\(_{82}\) ratio.
Figure 5. Absorption spectra of GdNP solutions (solid lines) and PEG-b-PAMA solutions (dotted lines) of the same concentrations, recorded in a 5 mm cell. The w/w ratio of PEG-b-PAMA/Gd@C$_{82}$ was 50 (a: 570 µg ml$^{-1}$ PEG-b-PAMA, 11.4 µg ml$^{-1}$ Gd@C$_{82}$) and 5 (b: 57 µg ml$^{-1}$ PEG-b-PAMA, 11.4 µg ml$^{-1}$ Gd@C$_{82}$). Dashed lines are difference spectra which represent Gd@C$_{82}$ absorption.

Figure 5 shows the optical absorption spectra of the resultant solutions. The spectra are dominated by a peak at 243 nm. Though the absorption of the GdNPs obtained using a w/w ratio of 5 is slightly broader than that of 50 (probably due to the small amount of aggregates), the complexes remained stably dispersed for over a month. This stability may be attributed to the strong adsorption of the PAMA segments on the Gd@C$_{82}$ cluster to form tethered PEG chains on the surface. In the vicinity of tumors, leaky vascular walls and immature lymphatic systems lead to the accumulation of high-molecular-weight drugs (the so-called enhancement permeation and retention effect). Hence, suitably sized nanoparticles (several tens of nanometers) can function as tumor-targeting drug carriers. The GdNPs obtained in this study meet this size criterion and thus show promise as tumor-tissue accumulation agents.

3.2. Cytotoxicity of GdNPs

While there are numerous reports concerning fullerene bioactivity, toxicity evaluation of fullerenes is hindered by their hydrophobic nature. Covalent derivatizations of fullerene compounds have been employed to improve solubility, but bioactivity often changes concurrently. As we could solubilize native fullerene and metallofullerene compounds without any chemical modification, toxicity evaluation was of great interest. Figure 6 shows the cytotoxicity results for GdNPs (w/w ratio = 50) determined by the WST assay using PEG-b-PAMA as a control. When a small amount of PEG-b-PAMA (3.8 mg mL$^{-1}$) was added to the culture media, the cell viability index increased, indicating rapid cell proliferation. Massia and Hubbell reported that tertiary amines accelerate cell growth analogously to lysine and arginine [38]. Small amounts of PEG-b-PAMA might exhibit the same effects as the amino acids because of the tertiary amines of PAMA segments. As the PEG-b-PAMA concentration was increased, cell viability decreased in a concentration-dependent manner. In contrast, GdNPs did not show any toxicity at up to 634 µM Gd which corresponds to a polymer concentration of 38 mg mL$^{-1}$. The lower toxicity observed for GdNPs might be due to the coordination of the PAMA segment to the Gd@C$_{82}$ cluster in the nanoparticle core. The GdNPs were much less toxic than previously reported Gd complexes at equal Gd atom concentrations [22, 39, 40].
3.3. Neutron irradiation of colon-26 cells with GdNPs

Since GdNPs showed extremely low toxicity \textit{in vitro}, we investigated the cell toxicity after low-energy neutron irradiation. Cell viability did not decrease upon the addition of GdNPs without neutron irradiation (vide supra) and neutron irradiation without GdNPs also did not show remarkable effects. Figure 7 shows the cell viability of colon-26 cells after neutron irradiation in the presence of GdNPs: when cultured cells containing GdNPs were irradiated with thermal and epithermal neutrons, cell viability decreased in a complex, dose-dependent manner. For example, a remarkable difference in cell viability was observed at high Gd concentrations—the cell viability decreased by 20% at 50 ppm Gd as compared to the unirradiated sample. To our knowledge, this is one of the first reports demonstrating the advantages of Gd@C\textsubscript{82} for NCT. Gd@C\textsubscript{82} is confirmed to show low toxicity without thermal and epithermal neutron irradiation and should be a suitable neutron capture agent for GdNCT.

We also calculated the absorbed dose of ionizing radiation in this experiment. According to reports, the absorbed dose when neutron irradiation was performed in cells without GdNPs was below 2 GyEQ, which is a low dose for the living body. However, the absorbed dose increased to over 15 GyEQ when cells with 100 ppm Gd atom were irradiated. The toxicity at 4 GyEQ is considerably higher than that at 2 GyEQ and would cause death in 50% of cases within 30 days. In this experiment, we have not confirmed the effect of particle size and cellular uptake of GdNPs because the neutron reaction does not depend on the particle size. Of course, the GdNP size may influence cellular uptake but this is not crucial for \textit{in vitro} experiments because the generated 2 MeV electrons are spread over several millimeters [41], and the distribution of gamma rays is even wider. These effects will be investigated later for \textit{in vivo} experiments and published elsewhere. This supplemental calculation further supports the use of GdNPs in neutron capture therapy. In addition, GdNPs have an advantage for bioimaging because Gd@C\textsubscript{82} can be used as a radiocontrast agent for MRI [33]. Neutron-capture therapy mediated by Gd@C\textsubscript{82} and the PEG block polymer complex will spur the development of a new approach to NCT assisted by bioimaging (to be published elsewhere).

### 4. Conclusions

In this study, we examined the neutron irradiation of colon-26 cells in the presence of GdNPs. The results indicate that GdNPs are a suitable neutron capture agent for GdNCT. The toxicity of the GdNPs is much reduced by complexing Gd@C\textsubscript{82} with PEG-b-PAMA block copolymer, which improves the usefulness of the Gd complex in both neutron capture therapy and as an MRI probe. Neutron irradiation of colon-26 cells dosed with GdNPs induces cell death, indicating the emission of gamma rays and conversion electrons upon the neutron capture reactions of \textsuperscript{155}Gd and \textsuperscript{157}Gd. The cytotoxicity data and the WST assay results suggest that Gd@C\textsubscript{82} has a high potential as a GdNCT agent. Because of the possibility of combining GdNCT with bioimaging, we anticipate that GdNCT will become a high-performance frontier therapy as compared to BNCT because biodistribution can be precisely monitored by MRI. Our results demonstrate that the rational material design of GdNPs holds promise for the future of GdNCT.

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