数理物質科学研究科　博士論文要約

専 攻 名　物性・分子工学
学籍番号　201235004
学生氏名　高　振宇
学 位 名　博士（工学）
指導教員　長崎幸夫

博士論文題目 Development of Boron-Cluster-containing Redox Nanoparticles for the Use in High Performance Boron Neutron Capture Therapy（ハイパフォーマンスな中性子捕捉療法に向けたホウ素クラスタ含有レドックスナノ粒子の開発）

1. Introduction

Boron neutron capture therapy (BNCT) is an external beam radiation treatment that is based on the capture reaction between thermal neutrons and nonradioactive isotope ^{10}B atoms, producing high linear-energy-transfer (LET) α particles and ^{7}Li nuclei with an average total kinetic energy of 2.34 MeV.[1-5] These high LET particles are reported to traverse 5–9 μm, similar to the diameter of one cell. Thus, cytotoxic effects are believed to occur in ^{10}B atom-containing tumor cells only after thermal neutron irradiation, without additional damage to the adjacent healthy tissues. Along the development of accelerator, which is smaller and more convenient to be set up in hospital compared with nuclear reactor, BNCT will potentially become a generic cancer therapy in the near future.

One of the most important issues of BNCT is to develop ^{10}B agents leading satisfactory therapeutic effect without strong adverse effect. However, owing to the low molecular weight (LMW), currently clinical applied ^{10}B agents are rapidly cleared from the circulatory system after administration. Thus, a high dose is generally required to achieve the effective ^{10}B concentration (>20 ppm) in tumors.[1] Furthermore, the nonspecific dispersion of LMW ^{10}B agents throughout the entire body also tends to increase the risk of both systemic toxicity and adverse effects during the thermal neutron irradiation.

To improve drug accumulation and retention tendency in the tumor site, nanoscale materials used to deliver ^{10}B agents for BNCT have attracted much attention.[6-9] Because they are only several tens of nanometers in size and are covered by biocompatible polymers such as poly(ethylene glycol) (PEG) as a shell, nanoparticles possessing high colloidal stability and non-biofouling characteristics tend to accumulate in tumor tissues as a result of the poorly aligned neovascularization and lack of effective lymphatic drainage in these areas, which is referred to as the enhanced permeability and retention (EPR) effect.[10] So far, most of the reported works that have used this strategy were focused on physically encapsulating boron-cluster compounds in liposomes.[6,7] However, because the encapsulation of these kinds of LMW compounds increases the osmotic pressure of liposome interior, encapsulated boron-cluster compounds tend to leak from the liposome into the blood stream, thus leading to a limited therapeutic effect.[8]

It was reported previously that inflammation of tumor microenvironment contributes to resistance to radiotherapy[11]. Nuclea factor-kappa B (NF-κB), which is one of transcription factors, plays a pivotal role in regulation of this kind of inflammation-based resistance and blocking of NF-κB to enter nucleus inhibited the adaptive radio resistance of tumor cells[12]. Furthermore, during BNCT, γ-ray emission due to the neutron capture reaction occurring in the tumor tissue also causes adverse effects, such as inflammation, by generating a large amount of reactive oxygen species (ROS).[13,14] Therefore, elimination of these original and BNCT-generated ROS is anticipated to increase the radio sensitivity of tumor cells and suppress ROS-induced adverse effects.

In this work, we synthesized a new anionic block copolymer, PEG-*b*-poly((closo-dodecaboranyl)thiomethylstyrene) (PEG-*b*-PMBSH), by introducing a negatively charged boron cluster, BSH, to the side chain of the poly(chloromethylstyrene) (PCMS) segment in the prepolymer PEG-*b*-PCMS. Because BSH is a hydrophilic and low-toxicity compound, it is much more suitable as a boron source than the hydrophobic carborane used in our previous work[15]. The total molecular weight of this block copolymer is approximately 7,000 Da; thus, it is anticipated to be excreted by renal filtration after the disintegration of the polyion complex (PIC) micelle, which was prepared in this study.[16] Our original nitroxide-radical-containing cationic block copolymer, PEG-*b*-poly(4-(2,2,6,6-tetramethylpiperidine-N-oxyl)aminomethylstyrene) (PEG-*b*-PMNT) was also synthesized by introducing 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (4-amino-TEMPO) as the side chain of the PCMS segment (Scheme 1), since the nitroxide radical in the TEMPO moiety strongly eliminates ROS. When the anionic PEG-*b*-PMBSH is coupled with the cationic PEG-*b*-PMNT, boron-cluster-containing redox nanoparticles (BNPs) are obtained via PIC formation. Note that because the BSH is conjugated to the block copolymer through a covalent bond, it is hard to leak BSH from the nanoparticle. The high charge densities and hydrophobic polystyrene segments are

expected to increase the colloidal stability under physiological conditions, without complicated chemical cross-linking or conjugation reactions. PEG-*b*-PMNT had been used as an antioxidant polymer drug (redox nanoparticle, RNP^N) and proved effective for oxidative stress-related diseases such as cerebral and renal ischemia-reperfusion injuries and cancer.[17-22] We have previously confirmed that RNP^N worked well to inhibit NF-κB expression in nucleus of tumor tissue and suppressed the inflammation[18]. Considering of this mechanism, suppression of NF-κB activation might also improve therapeutic effect of BNPs because of PEG-*b*-PMNT as a counterpart of the BNP. Thus, these BNPs are anticipated to be a high-performance nanomedicine for BNCT, with the ability to lower the adverse effects by scavenging ROS as well as by effectively damaging the tumor cells.

2. Synthesis and Characterization of PEG-*b*-PMBSH

The boron-cluster-conjugated anionic block copolymer PEG-*b*-PMBSH was synthesized for the first time by introducing a clinically applied ^{10}B agent (BSH) to the polystyrene segment of PEG-*b*-PCMS. Since the nucleophilicity of the sulfanyl group of BSH is lower than that of the conventional thiols,[23] and rather similar to that of the hydroxyl groups, sodium hydride was employed as the metalation agent to convert the thiolate anion, which could then react with the chloromethyl group in PCMS, resembling the Williamson ether synthesis method. The increased broad signal appearing from 0.5 to 2.0 ppm is assignable to B-H in the boron cluster,[23] confirming the immobilization of BSH in the polymer.

3. Preparation and Characterization of BNPs

The BNPs were prepared by mixing PEG-*b*-PMBSH and PEG-*b*-PMNT in phosphate buffer solution with various concentrations of boron and various molar ratios of polyanion/polycation. The mixtures under the present preparation conditions were transparent, and the main products were micellar structures. The concentration of BNPs affected their dispersion stability and caused aggregation under low concentration conditions. An almost completely unimodal distribution of nanoparticles (of several tens of nanometer size) was observed by dynamic light scattering (DLS). The zeta potential of this complex was close to zero, suggesting complete shielding of the charges of BSH by the PEG outer layer.

To obtain further information on the colloidal stability of the BNPs, after incubation in mice plasma solution or phosphate-buffered saline (PBS) solution containing 10% fetal bovine serum (FBS) for 48 h at 37 °C, size exclusion chromatography (SEC) analyses of the BNPs were performed and monitored with a UV detector at 220 nm. A solution of BNPs in PBS was used as a control. No decrease of intensity or shift of peak was observed in the presence of mice plasma or 10% FBS. Eluates of BNPs in mice plasma solution were collected every 30 s and subjected to ICP-MS and DLS analyses. Neither a peak related to the aggregates nor disassembly of the BNPs was observed, confirming their highly stable character even in serum proteins. Considering the results above, BNPs have very high colloidal stability affording them for the further *in vitro* and *in vivo* study.

4. Cytotoxicity and Cellular Uptake of BNPs

The cytotoxicity of the BNPs was evaluated by WST-8 cell proliferation assay kit using mouse colorectal carcinoma cells (C-26, mouse cell line derived from rectal cancer, BALB/c strain). Generally, cationic polymers and nanoparticles with a positive shell are strongly toxic because they readily aggregate with anionic serum proteins and disrupt the plasma membrane. For example, the half maximal inhibitory concentration (IC_{50}) values of poly(ethylenimine) at different amine concentrations were 3.7 μg/mL (amine: 0.086 mM, MW = 25 kDa) and 12.4 μg/mL (amine: 0.288 mM, MW = 1.8 kDa).[24] It is interesting to note that BNPs did not show cytotoxicity at all in a relative high concentration of ^{10}B. It may be because of their neutral surface and high colloidal stability.

As mentioned above, the high LET α particles and ^{7}Li nuclei generated from the neutron capture reaction of ^{10}B can traverse 5–9 μm, which corresponds with one cell diameter. We strongly believe that the enhancement of cellular uptake of the ^{10}B agent by tumor cells and its suppression in normal cells are pivotal for satisfactory BNCT, in order to provide more effective damage to the tumor cell while simultaneously suppressing the adverse effects on normal tissues caused during the treatment. We evaluated the cellular uptake of BNPs in both the C-26 cell line and human aortic endothelial cell line (HAEC) and calculated the uptake ratio. The LMW ^{10}B agents BPA-fructose complex and BSH were used as controls. Because BSH is a divalent anion, it is barely able to access the cellular membrane, resulting in low cellular uptake. No tumor selectivity for the internalization of BSH was observed. On the other hand, with the BPA-fructose complex, the tumor cells showed elevated and selective internalization of BPA, which is ascribed to the preferred cellular uptake (especially by tumor cells) of amino acid derivatives of BPA.[5] The selective internalization of BPA to tumor cells was 2.5-fold higher than to normal cells. The preferential internalization of BNPs to tumor cells relative to normal HAECs was 3.3-fold, much higher than that of BPA. This is probably due to the enhanced solubilization of the nanoparticles to the membrane lipid bilayer through the PEG fusion process. PEG-modified derivatives are widely known to undergo enhanced cellular uptake in many types of cancer cells.[25]

5. Pharmacokinetics of BNPs in Tumor-bearing Mice

The accumulation tendency of boron species in the tumor environment is quite important in practical treatment *in vivo*. Thus, a pharmacokinetics study of the BNPs was performed to investigate their blood circulation tendency and tumor accumulation character in C-26 tumor-bearing BALB/c male mice. The ^{10}B concentration in circulating blood decreased slowly after intravenous (i.v.) injection (via the tail vein) of BNPs. The long circulation tendency of BNPs is important to increase their accumulation in the tumor site, because repeated access to the tumor neovascular wall increases leakage in the area via the EPR mechanism. The ^{10}B concentration in tumor tissues reached high level at 48 h after administration, and remained at this level until 72 h without significant change. In contrast, the LMW BSH was excreted rapidly after 1 h, and almost disappeared in the blood circulation after 24 h without specific tumor accumulation.[15] Such increased accumulation, prolonged retention in tumor environment, and selective internalization to tumor cells of the BNPs might improve the therapeutic effect of thermal neutron treatment, which is described later.

6. Enhanced Boron Neutron Capture Therapeutic Effect by BNPs

Thermal neutron irradiation was carried out to evaluate the therapeutic effect of the BNPs in C-26 tumor-bearing BALB/c male mice. BPA-fructose complex, RNP^N, and PBS administration followed by thermal neutron irradiation, and PBS administration without irradiation were used as controls. In both groups of BNPs administration, the tumor/blood (T/B) ratio of ^{10}B concentration (one of the most important factors) was higher than that in the BPA-fructose complex group. A higher T/B ratio implies lower adverse damage to the circulation system and normal tissues during irradiation, because of the lower amount of ^{10}B atoms in the blood vessels.

PBS-treated group showed slight tumor suppression effect after thermal neutron irradiation compared to the unirradiated group, which is probably because of an effect of γ ray mixed in the neutron ray from the nuclear reactor. It is interesting to note that the BNP-treated group, with much lower ^{10}B concentration in the tumor, suppressed tumor growth to the same degree as the BPA-fructose complex group.

The therapeutic effect of BNPs on a relatively large tumor model was then investigated. That is, the ^{10}B agents were administered to tumors of an average volume of 340 mm^3. All ^{10}B samples were administered to tumor-bearing mice by i.v. injection via the tail vein 3 d before the thermal neutron irradiation, except for BSH, which was given 1 h before the irradiation because of its rapid excretion. In the BNP-treared group, 5 ppm of ^{10}B was observed in the tumor tissues, whereas 15 ppm accumulated in the tumor tissues administered BSH. Almost no tumor growth was observed in the BNP-treated group, regardless of the low ^{10}B concentration in the tumor tissues. It is again observed that saline-treated group showed a slight suppression effect of tumor growth after thermal neutron irradiation, because of γ ray irradiation. The relative body weight of the mice in the BNP-treated group kept increasing after a transient period, indicating that BNPs did not have significant systemic toxicity.

7. Suppression of Adverse Effects After BNP-assisted BNCT

BNCT is considered as a novel cancer therapeutic with low adverse effects, because the thermal neutron ray is a benign beam source that does not cause the direct strong ionization damage to healthy cells, which is commonly observed in radiotherapy using x ray and γ ray. However, γ rays are still emitted by the neutron capture nuclear reaction in tumor cells during the irradiation and cause oxidative damage to healthy tissues by generating ROS,[13,14] increasing the leukocyte level. Both the BPA-fructose complex and PBS-treated groups presented significantly high leukocyte levels after irradiation, compared with the control group and the PBS-treated group without irradiation (PBS-C). On the other hand, the BNP and RNP^N-treated groups presented almost the same leukocyte level as the controls, indicating that the ROS scavenger might work to suppress the increasing in leukocyte level. Let us emphasize again that the BNPs worked well to suppress tumor growth despite the very low ^{10}B concentration in tumor tissues, even though it eliminated generated ROS, indicating that the observed suppression of tumor progression was not governed by ROS generation but rather by the direct attack of the generated active species (such as α ray and ^7Li nuclei) by the nuclear reaction between the BNPs and thermal neutrons. The generated ROS cause inflammation and adverse effects to the entire body. On the basis of these results, it is concluded that the ROS scavenging ability installed in the BNPs helped to enhance the therapeutic effect and suppress inflammation, which is one of the main adverse effects of BNCT caused by ionization during the irradiation.

8. Discussion

With BNPs, a low ^{10}B concentration in tumor tissues led to an ideal tumor suppression effect. On the other hand, satisfactory therapeutic effect in the BSH-treated group was not observed. The higher and specific cellular uptake into tumor cells of BNPs might contribute effectively to this satisfactory therapeutic effect. In addition, the high tumor retention of ^{10}B atoms during the 40 min irradiation in the BNP-treated group might guarantee the required ^{10}B concentration in tumor tissue, even with the lower initial boron dose. In the case of LMW ^{10}B agents (BPA and BSH), the ^{10}B atoms in tumor tissue kept decreasing as a result of rapid excretion, and thus a higher boron dose might be required. From these results, BNPs indeed damaged the tumor cells, and suppressed the tumor growth and metastasis, as well as suppressed adverse effects through ROS scavenging activity after thermal neutron irradiation. We strongly believe that the BNP is a quality candidate as a boron delivery system for satisfactory BNCT performance.

9. Summary

- ✓ We have successfully synthesized boron-cluster-containing block copolymer and prepared the boron-cluster-containing redox nanoparticles (BNPs) with 36 nm size.
- ✓ BNPs show high colloidal stability under physiological conditions, low cytotoxicity without irradiation, specific cellular uptake into tumor cells compared to normal cells, specific tumor accumulation tendency and longer tumor retention time compared with LMW ^{10}B agents.
- ✓ Satisfactory therapeutic effect by low ^{10}B dose and suppression of adverse effect were confirmed in BNP-assisted BNCT.

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