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研究課題名（和文）Reactive oxygen species-sensitive prodrug micelles

研究課題名（英文）Reactive oxygen species-sensitive prodrug micelles

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研究成果の概要（和文）：本研究は、活性酸素種（ROS）応答性をもつ高分子ミセルを合成し、腫瘍特異的な抗がん剤の輸送を可能とする薬物輸送システムの開発を目的とした。当初、ROS応答性をもつボロン酸エステル含有モノマーの重合により、両親媒性ジブロックコポリマーを合成する予定であったが、重合制御が難しい上に、得られたポリマーが容易に加水分解されてしまった。そこで、フェニルボロン酸基をもつモノマーを水溶液中で分散重合することによりナノ粒子を合成した。得られた粒子は、過酸化水素の消去能をもつことを確認した。さらに、この粒子に分解性の結合を介してドキソルビシンを担持し、癌細胞における毒性を確認した。

研究成果の概要（英文）：Cancer is one of the leading causes of death in the developed world. In order to deliver anti-cancer drugs to the tumor site polymeric nanoparticles (NPs) have been developed. The characteristic properties of the tumor such as the presence of reactive oxygen species (ROS) and decreased pH can be used as a stimulus to release the drug from the NPs. In this project the applicant aimed to prepare boronic acid-containing micelles that could release a drug in response to ROS. Due to problems with this approach a NP platform, also containining boronic acid groups was developed using a different approach. These NPs were able to scavenge H2O2. Furthermore these NPs could be modified using the Suzuki coupling reaction to incorporate drug moleules. The drug doxorubicin was conjugated via an acid sensitive hydrazone bond. These doxorubicin-NPs were cytotoxic in two different cancer cell lines and should enable drug release in the acidic tumor microenvironment.

研究分野：高分子化学

キーワード：ナノ粒子　ボロン酸　活性酸素　ドラッグデリバリー

１．研究開始当初の背景

Cancer is one of the leading causes of death in the developed world. For delivering therapeutic drugs to the tumor tissue the concept of polymeric nanoparticles (NPs) has proven to be a very powerful approach improving pharmacokinetics mainly because of the passive accumulation of these polymeric micelles into tumors due to their small size of about 50-100 nm by the EPR effect. Over the years it has become clear that reactive-oxygen species (ROS) play an important role in tumor progression and metastasis. Reactive oxygen species like superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are known to be involved in cancer initiation and progression. One of the processes in which ROS has an important role is the induction of growth factors for angiogenesis, the formation of new blood vessels. This vascularization is important for tumor growth for supplying the cancer cells with nutrients. Apart from the fact that cancer cells produce ROS themselves, macrophages that have been directed by the immune system to the tumor site also contribute to ROS by producing H_2O_2. ROS species are therefore both present intra- and extracellular at the tumor site. Apart from the presence of ROS the tumor site is also characterized by an acidic pH.

２．研究の目的

In this proposal the applicant aimed to design polymeric prodrug micelles that can release an anti-cancer drug in response to ROS in the tumor microenvironment and inhibit tumor growth and spreading.

To achieve this, a polymer having boronic acid ester groups was proposed. Boronic acid esters R'-$B(OR)_2$, with R' being any organic group and R an alkyl group, are compounds having a boron-carbon bond that is susceptible towards oxidation. Reaction with H_2O_2 yields the corresponding more hydrophilic alcohol R-OH. This hydrophobic to hydrophilic transition might be a viable concept to direct disassembly of a prodrug micelle at the tumor site by ROS with concomitant drug release. Apart from improving drug release at the tumor site the ROS-scavenging activity of the micelles has the potential of interfering with cellular processes such as angiogenesis in the tumor directly. Parallel with this micelle approach boronic acid nanoparticles (NPs) prepared by a surfactant free method were also studied. With these particles ROS scavenging could be studied as well. Furthermore the presence of phenylboronic acid group should enable functionalization of the nanoparticles by the Suzuki reaction. By conjugating the drug by an acid-sensitive hydrazone bond the lower pH encountered in the tumor tissue could be exploited to deliver an ant-cancer drug.

３．研究の方法

3.1.1 Block copolymer synthesis

N-vinyl pyrrolidone (NVP) was freed from inhibitor by passing over silica. Pinacol vinylboronic ester (PVB) was freed by inhibitor by distillation under reduced pressure. AIBN was recrystallized from MeOH. The RAFT agent cyanomethyl methyl(phenyl)carbamodithioate was used as received. Poly-NVP was prepared by polymerizing NVP and the RAFT agent in the presence of AIBN (RAFT/AIBN=5) in CH_3CN at 60°C. The polymer was recovered by precipitation in Et_2O and used as a macro RAFT for polymerizing PVB with the same RAFT/AIBN molar ratio in CH_3CN at 60°C. After precipitation in Et_2O this yielded the block copolymer. The macro RAFT agent poly(ethylene glycol)-xanthate ethyl ester was prepared and also used to polymerize the PVB monomer.

3.1.2 GPC and ^1H NMR block copolymers

The synthesized polymers were characterized by ^1H NMR to determine the block copolymer composition. Furthermore gel permeation chromatography was done using Shodex or TSK gel size exclusion columns using 100 mM LiCl in DMF or $CHCl_3$ as the eluent respectively.

3.1.3 DLS, TEM and ^1H NMR micelles

The polymers were dissolved at 50 mg/mL in NMP and mixed 1:9 with water. Organic solvent was removed by dialysis using MWCO 1000. The size of the micelles was measured on an Otsuka dynamic light scattering (DLS) machine. After adsorbing on to carbon grids samples were negatively stained with tungstate and measured by transmission electron microscopy (TEM). Micelle solutions were lyophilized dissolved in $CDCl_3$ and measured by ^1H NMR.

3.2.1 Boron nanoparticle (B-NP) synthesis

3-Aminophenylboronic acid acrylamide (APBA) was prepared from acryloyl chloride and 3-aminophenylboronic acid in

a THF/water mixture in the presence of $NaHCO_3$. After solvent removal, stirring with EtOAc for 4 h and aqueous washing the crude monomer was recrystallized from water. PEG-acrylamide was prepared from hydroxyl-terminated PEG as follows: PEG-OH was reacted to give the PEG-mesylate that was reacted with potassium phthalimide in DMF to yield PEG-phthalimide. Deprotection with hydrazine yielded the PEG-amine that was reacted with acryloyl chloride to yield PEG-acrylamide. The cross-linker methylene bisacrylamide (MBEM) and radical initiator ammonium persulfate (APS) were recrystallized from MeOH and EtOH/H_2O respectively. Nanoparticles were prepared at different monomer ratios in phosphate buffer under N_2 at 70°C. After 24 h the solutions were dialyzed with MWCO 2000.

3.2.2 DLS, AFM, SEM and TEM of B-NPs
After dialysis particles were measured by DLS. Micelle solutions were also used to prepare samples for atomic force microscopy (AFM), scanning electron microscopy (SEM) and TEM.

3.2.3 Time course B-NP formation
The synthesis was started and at the indicated time points terminated by cooling in liquid N_2. After thawing to room temperature DLS was measured. The solution was then analyzed by HPLC with a reversed phase column to determine the amount of remaining monomer.

3.2.4 ROS-scavenging properties B-NPs
The nanoparticles were mixed with 10 μM H_2O_2 (aq) and incubated with different B-NP concentrations. Remaining H_2O_2 was then measured using the Amplex Red assay. The reactive oxygen scavenging properties was then compared with H_2O_2 (aq) incubated in the absence of B-NPs.

3.2.5 Model Suzuki reaction
For this a boronic acid model compound was prepared by reacting 3-aminophenylboronic acid with propionic acid using EDC. A new catalytic palladium (Pd) system was developed for doing aqueous Suzuki reactions. Na_2PdCl_4 was mixed with the phosphine ligand (PPh_2PhSO_3Na) and added to the reaction mixture containing the boronic acid, 4-iodo or 4-bromobenzoic acid and K_2CO_3. The reaction mixture was then placed at 70°C. After 24 h a small portion was quenched with $NaHSO_4$ (aq) and lyophilized. ^1H NMR in DMSO was done to determine the extent of reaction. The remainder of the reaction mixture was acidified, filtered, dissolved in acetone and evaporated to determine the isolated yields.

3.2.6 Suzuki coupling with B-NPs
The nanoparticles were reacted with 4-iodo/benzoic acid, 4-iodo benzaldehzyde and 4-(bromoethyl)benzoic acid using 1% Pd at 70°C. Quantitative conversion of the boronic acid was confirmed using the fluorescent dye Alizarin Red 2S. Furthermore after lyophilization infrared (IR) was done to confirm the presence of carboxylate and aldehyde groups.

3.2.7 Preparation doxorubicin-NPs
Commercial available 4-iodobenzoic acid and 4-(bromoethyl)benzoic acid were reacted with Boc-protected hydrazine in the presence of EDC. The compounds were purified by silica column chromatography and characterized by ^1H NMR. These compounds were reacted with B-NPs using the Pd catalyst. After filtering and dialysis the particles were treated with HCl (aq) and lyophilized to yield the NPs with an alkyl and phenyl linker. The solid was mixed with doxorubicin and a catalytic amount of CF_3COOH in DMF. After 24 h at 60°C the free drug was separated from the doxorubicin-NPs by size exclusion on Sephadex LH20 in DMF. The fractions containing NPs were combined and dialyzed against PBS pH7.4 to remove DMF. The particles were then characterized by DLS.

3.2.8 Cellular toxicity doxorubicin-NPs
To evaluate the toxicity of doxorubicin -NPs they were incubated with MCF7 and HT29 cells and their toxicity evaluated by the MTT assay after incubation for 2 days.

４．研究成果
4.1 Boronic acid micelles
Using the RAFT methodology we prepared three different polymers differing in their relative block size according to ^1H NMR: $NVP_{55}PVB_{42}$, $NVP_{55}PVP_{69}$ and $NVP_{108}PVP_{33}$. Whereas ^1H NMR confirmed the polymerization of both monomers, i.e. the absence of vinyl protons and broad signals of the other protons GPC could not be used to confirm the formation of block copolymers. The polymers behaved strange with the NVP and NVP_x-PVB_y polymers eluting at the same time. However all

three polymers gave micelles when dispersed in water with a size of 79.1, 100.9 and 88.8 nm according to DLS, confirming the presence of block copolymers. The spherical shape of the micelles was confirmed using TEM after negatively staining. Unfortunately the PVB hydrophobic block turned out not to be hydrolytically stable and ^{1}H NMR revealed that about 50% of the boronic acid esters were hydrolyzed after only 2 days.

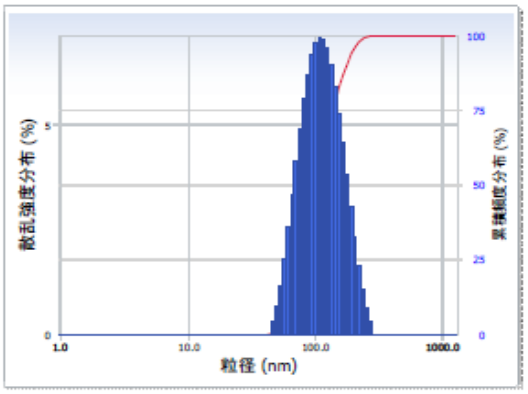

Figure 1. Dynamic light scattering data for micelles prepared from $NVP_{55}PVP_{69}$.

To tackle this problem a more hydrophobic boron monomer was used. Furthermore to solve the problem with the physiochemical characterization of the polymers it was decided to replace the hydrophilic block PVP with PEG. It was hoped that this also could help in stabilizing the hydrophobic core. A PEG-xanthate RAFT agent was successfully prepared, but unfortunately, this RAFT agent was not capable of polymerizing vinyl boronic acid monomers. Since parallel with the micelle approach the applicant had successfully synthesized boronic acid-containing nanoparticles by a surfactant-free polymerization method efforts were focused on evaluating the ROS-scavenging properties of these NPs. Furthermore since the NPs displayed phenylboronic acid groups in contact with the aqueous phase the well-known Suzuki reaction was investigated as a means to conjugate anti-cancer drugs via an acid-labile linker. In this way the acidic pH at the tumor site could be exploited to induce drug release.

4.2.1 Preparation boronic acid NPs

By simply heating a mixture of APBA, MEBA and PEG-acrylamide at 70°C in the presence of APS under nitrogen NPs were prepared as confirmed by DLS. The effect of each monomer on particle size was investigated. APBA and PEG-acrylamide both had a strong effect whereas the MEBA cross-linker did not show any significant effect. With increasing APBA concentration the particle size increased and with increasing PEG-acrylamide concentration particle size decreased. By adjusting the monomer concentrations the NP size could be tuned in the range 80-250 nm, a size range well suited for drug delivery application.

4.2.2 Structure and formation NPs

Interestingly AFM, SEM and AFM revealed an unique framboidal morphology not described before in the literature. To understand how these NPs formed the evolution of NPs was followed as function of time by DLS, AFM and HPLC. DLS indicated that after about 10 min NPs formed about 120 nm in diameter that slowly increased size for about 1 h to 150 nm. HPLC idicated that after about 10 min 50% of APBA was consumed with only 5-10% of the cross-linker and PEG-acrylamide incorporated. AFM revealed at early time points the formation of small (about 20 mm) spherical particles that grew into framboidal structures at longer time points. From this the following model is proposed. At first APBA-rich particles form having some PEG incorporated. This PEG avoids the particles to fuse, what normally would occur in dispersion polymerization, leading to the observed framboidal morphology.

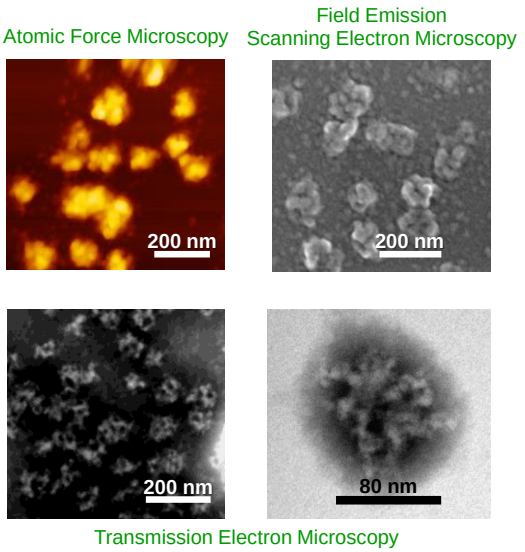

Figure 2. Atomic force microscopy, scanning electron microscopy and transmission electron spectroscopy images of the B-NPs

4.2.3 ROS-scavenging properties B-NPs

Since the NPs contain boronic acid groups its reaction with H_2O_2 was investigated. H_2O_2 is a ROS-species formed by the enzyme superoxide dismutase acting on the other ROS species superoxide. The B-NPs were left in the presence of H_2O_2 and the amount of remaining H_2O_2 was measured using a fluorescent method. The B-NPs were able to scavenge H_2O_2 in a dose-dependent matter showing its potential in ROS scavenging applications.

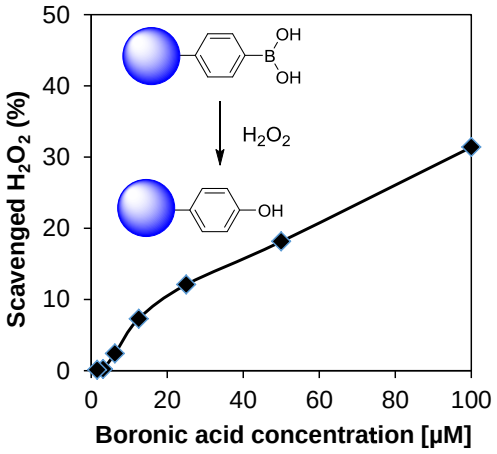

Figure 3. ROS-scavenging properties of the B-NPs measured with the Amplex red assay.

4.2.4 Suzuki reaction NPs

To test the possibility of further functionalizing the NPs we investigated the Suzuki coupling reaction between phenylboronic acid and iodo/bromo phenyl derivatives in the presence of a palladium catalyst. Since not many reports on the Suzuki reaction in water has been reported we used a new catalytic Pd catalyst and used a model reaction between 3-propionamidophenylboronic acid and 4-iodo/bromobenzoic acid. We found this system to be very efficient for 4-iodobenzoic acid reaching full conversion at 0.1 mol% Pd at room temperature. In case of 4-bromobenzoic acid the system was less active requiring 0.01 mol% Pd at 70°C. For this reason the temperature was fixed at 70°C and to take into account the lower boronic acid concentration in the NP solution the Pd catalyst loading was fixed at 1%. Using these conditions NPs could be functionalized with different coupling partners. Full conversion was confirmed by the absence of phenylboronic acid groups after reaction using a phenylboronic acid-specific fluorescent detection dye. Furthermore IR showed the characteristic bands of the C=O stretching vibration of carboxylic acid and aldehyde groups at around 1700 cm^{-1}.

Table 1. Suzuki coupling model reactions at different temperatures and Pd loadings.

Pd/B [mol %]	T [°C]	X	Conversion [%]	Yield [%]
1	70	I	100	94
0.1	70	I	100	79
0.01	70	I	100	93
1	25	I	100	98
0.1	25	I	100	91
0.01	25	I	47	n.d.
1	25	Br	78	n.d.
1	70	Br	100	98
0.1	70	Br	100	99
0.01	70	Br	100	100

4.2.5 Doxorubicin NPs

Next we investigated the conjugation of the anti-cancer drug doxorubicin to the NPs. As stated in the background the tumor microenvironment is also characterized by a low pH. Since doxorubicin contains a ketone group we used a hydrazone linkage to conjugate it to the NPs. This hydrazone bond is hydrolyzed at low pH but is stable at physiological pH. We therefore prepared two different NPs differing in the hydrazide structure denoted as alkyl and phenyl. The Suzuki coupling reaction was used to introduce these protected hydrazides that after acid cleavage were reacted with doxorubicin. DLS showed both particles to be about 150 nm in diameter. The doxorubicin-NPs were then tested in two different human cancer cell lines: MCF7 breast cancer and HT29 colon cancer.

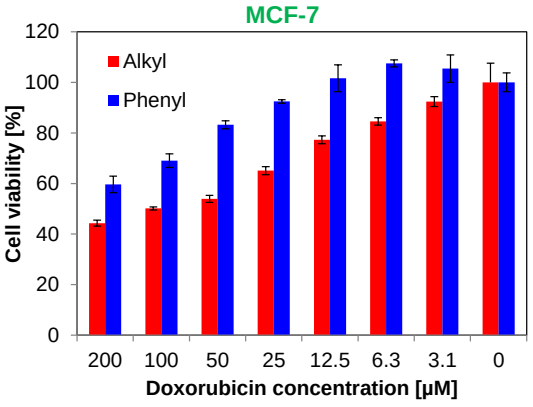

Figure 4. Cell viability in response to different doxorubicin-NPs in MCF7 human breast cancer cells.

After 2 days cell viability was measured by the MTT assay. It was shown that the NPs are more toxic in breast cancer cells. Furthermore the NPs with the alkyl linker appeared to more toxic than the phenyl linker.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　　件）
Not applicable.

〔学会発表〕（計　3 件）
1. A.J. van der Vlies, U. Hasegawa, Functionalization of boronic acid nanoparticles for drug delivery application. 第 64 回高分子学会年次大会, 2015 年 5 月, 札幌コンベンションセンター（北海道　札幌市）
2. A.J. van der Vlies, U. Hasegawa, Functionalization of boronic acid nanoparticles by Suzuki coupling for drug delivery application, The 2015 Materials Research Society Spring Meeting, 2015 年 4 月, サンフランシスコ（アメリカ）
3. A.J. van der Vlies, U. Hasegawa, Suzuki coupling-functionalized boronic acid nanoparticles for drug delivery, 26th Annual Conference of the European Society for Biomaterials, 2014 年 9 月, リバプール（イギリス）

〔図書〕（計　　件）
Not applicable.

〔産業財産権〕
○出願状況（計　　件）

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発明者：
権利者：
種類：
番号：
出願年月日：
国内外の別：

○取得状況（計　　件）

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発明者：
権利者：
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〔その他〕
ホームページ等

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