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厚生労働科学研究費補助金

医療機器開発推進研究事業

固形がんの標的治療とその治療効果のMRIによる追跡を可能にする  
診断-治療機能一体型DDSの創製

平成23年度 研究報告書

研究代表者 西山 伸宏

平成24（2012）年 5月

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診断-治療機能一体型DDSの創製

研究代表者 西山伸宏 東京大学大学院医学系研究科臨床医工学部門 准教授

研究要旨

がん組織に選択的に集積し、がんのMRI診断に広く利用できるMRI造影剤が望まれているが、そのような造影剤は未だ開発されていないのが現状である。本研究では、前年度までに抗がん剤DACHPtとMRI造影剤Gd-DTPAを同時に搭載したDACHPt/Gd-DTPA内包ミセルを開発し、マウス膵臓がんモデルにおいて、抗がん剤による治療効果をMRIによって追跡できることを明らかにしてきた。しかしながら、本システムは投与量に関して、制がん剤の最大耐用量に基づく制限があり、鮮明な固形がんイメージングを達成するためには制がん活性を持たないGd-DTPA内包ミセルを開発する必要があるものと考えられた。そこで本年度は、新たな分子設計に基づいてGd-DTPAを内包した高分子ミセルを構築し、その基本性能を明らかにした。

A. 研究目的

がん組織に選択的に集積し、がんのMRI診断に広く利用できるMRI造影剤が望まれているが、そのような造影剤は未だ開発されていないのが現状である。また、がん化学療法においては、その有効性を的確に把握する必要があり、その目的においてMRIは有効な手段であると考えられる。近年、Gdのデリバリーの為のDDSが活発に開発されているが、固形がんの選択的造影に成功した例は数少なく、すべてのシステムにおいてGdがDDS担体に安定な共有結合で導入されている為、Gdの蓄積毒性が問題となる。そこで本研究では、Gd-DTPAと白金の可逆的な錯体形成を利用してGd-DTPAを内包した高分子ミセルを開発している。このようなシステムは、Gd-DTPAがDDS担体から最終的には放出され、体外に排泄されるために、安全性面において極めて優れていると考えられる。

本研究では、前年度までに、研究代表者である西山が過去に開発を行い、現在、第一相臨床治験中のオキサリプラチン活性体(DachPt)を内包する高分子ミセルに、MRI造影剤のGd-DTPAを搭載し、DachPtによる治療効果をMRIによって追跡できる診断-治療機能一体型DDSの開発を行ってきた。本システムの基本性能をマウス膵臓がんモデルを用いて検証した結果、制がん剤による治療効果をMRIによって追跡できることが明らかになり、Theranostic Nanodeviceのコンセプトを実証することができた。しかしながら、本システムは投与量に関して、制

がん剤の最大耐用量に基づく制限があり、鮮明な固形がんイメージングを目的として投与量を上げるためには、制がん活性を持たないGd-DTPA内包ミセルを開発する必要があるものと考えられた。

そこで3年計画の最終年度にあたる本年度は、分担研究者のCabralの分担研究報告書に記載したように、制がん活性を持たないヘキサクロロ白金酸( $H_2PtCl_6$ )と生分解性を有することにより蓄積毒性を示さないPEG-PAsp(DET)の金属錯体形成により高分子ミセルを形成させ、Ptの可逆的な金属錯体形成を介してMRI用陽性造影剤のGd-DTPAを内包させた系を開発した(図1)。

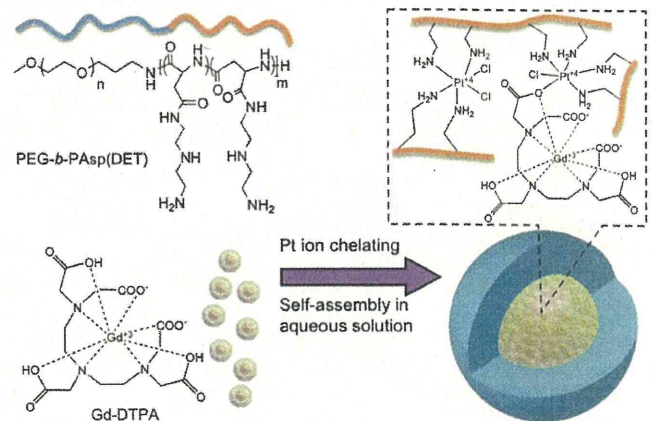


図1. 白金の金属錯体形成を利用してGd-DTPAを内包した高分子ミセル

具体的な実験としては、Gd-DTPA内包ミセルが毒性が低いMRI造影剤であることを明らかにするため

に細胞毒性評価を行い、さらにMRI造影剤としての性能を明らかにするためにパルスNMRによるT1緩和能の測定を行った。さらに、マウス大腸がんの皮下移植モデルを用いて、Gd-DTPA内包ミセルの体内動態を明らかにし、固形がんのMRイメージングを行った。

## B. 研究方法

### 1. Gd-DTPA内包ミセルの細胞毒性評価

Gd-DTPA内包ミセルならびにそれらの構成成分と正常ヒト臍帯静脈内皮細胞 (HUVEC) を培地中で72時間培養し、細胞生存率をCCK-8 assayによって評価した。

### 2. Gd-DTPA内包ミセルのT1緩和能の測定

Gd-DTPAおよびGd-DTPA内包ミセルのT1緩和能をパルスNMR (JNM-MU25A, JEOL) を用いたファントム実験により測定した。T1緩和能 ( $r_1$ ) は以下の式より算出した。

$$1/T_1 = 1/T_{10} + r_1 [\text{Gd}]$$

T<sub>1</sub>: スピン-格子緩和時間 (縦緩和時間)

### 3. Gd-DTPA内包ミセルの体内動態評価

マウス大腸がんC26細胞をBALB/cマウス (♀, 6週齢) の皮下に直接注入することによって固形がんモデルマウスを作製した。このマウスに対して、Gd-DTPAおよびGd-DTPA内包ミセルをi. v. 投与し (Gd換算で78 μg/mouse)、一定時間経過後に血漿と主要臓器ならびに固形がんを摘出した。その後、得られた生体組織を90% HNO<sub>3</sub>中で加熱することによって溶解し、1% HNO<sub>3</sub>溶液に調製した後にPt量をICP-MSによって定量した。

### 4. 蛍光X線によるPt、Gdのがん組織への集積性の評価

上述の固形がんモデルマウスに対してGd換算で100 μg/mouseのGd-DTPA内包ミセルを投与し、24時間後の凍結切片のSpring-8を用いた蛍光X線分析 (μ-SR-XRF) を行った。

### 5. 固形がんモデルのMRイメージング

上述の固形がんモデルマウス (腫瘍サイズ100 mm<sup>3</sup>) に対して、0.02 mmol/kgのGd-DTPAおよびDachPt/Gd-DTPA内包ミセルを投与し、1-T MRI (Aspect Imaging) によってマウスの腹腔部のMRイメージングを行った。MRイメージングに関しては、ファントムをリファレンスとして投与から10分毎に4時間までの画像を取得した。

## C. 研究結果

### 1. Gd-DTPA内包ミセルの細胞毒性評価

Gd-DTPA内包ミセルならびにそれらの構成成分の

HUVECに対する細胞毒性を評価した (図2)。その結果、H<sub>2</sub>PtCl<sub>6</sub>は0.1mg/mLにおいて毒性を示したものの、Gd-DTPA内包ミセルならびにそれらの構成成分はHUVECに対して細胞毒性を示さないことが明らかになった。

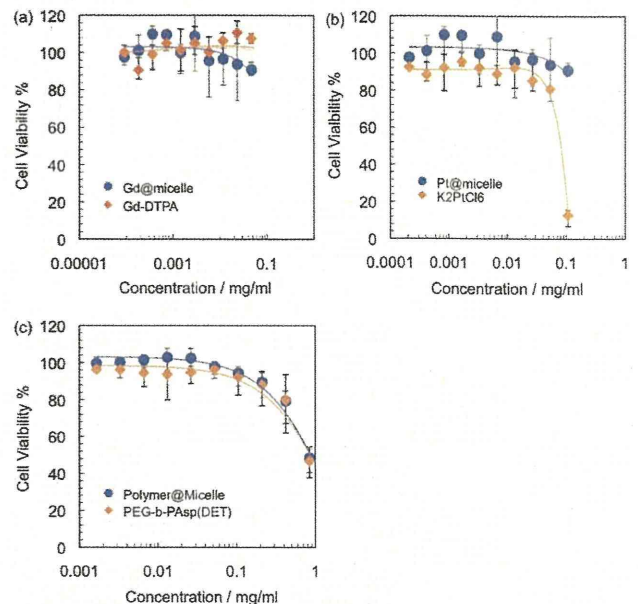


図2. HUVECに対するGd-DTPA内包ミセルおよびそれらの構成成分の細胞毒性評価 (72時間培養)

### 2. Gd-DTPA内包ミセルのT1緩和能の測定

Gd-DTPA内包ミセルのT1緩和能をパルスNMRを用いたファントム実験により評価した (図3)。その結果、Gd-DTPA内包ミセルは、Gd-DTPAの13.1倍のT1緩和能を有することが明らかとなった。

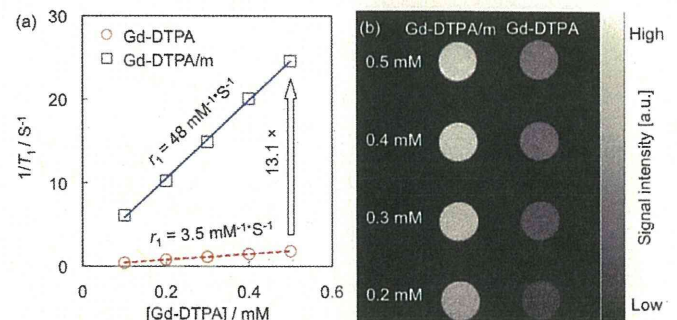


図3. パルスNMRを用いたファントム実験によるGd-DTPA内包ミセルのT1緩和能の測定

### 3. Gd-DTPA内包ミセルの体内動態評価と固形がんモデルのMRイメージング

C26細胞の皮下移植モデルマウスにおけるGdおよびPtの血中滞留性、Gdの固形がんへの集積性、正常臓器とがんのGdの集積比をGd-DTPA内包ミセルとGd-DTPAと比較した (図4a-d)。その結果、Gd-DTPAと比較してGd-DTPA内包ミセルはGdの血中滞留性と固形がんへの集積を高め、がんへの選択的な集積を実現することが明らかになった。

固形がんの凍結切片のμ-SR-XRFによって得られ

たFe、Gd、Ptの元素マッピングの結果を図4eに示した。ここで、Feは、ヘム鉄によるものであり、その分布は血流の分布を反映している。このことを考慮すると、GdとPtはどちらもがん組織に集積し浸透していることが分かる。

さらに、1T-MRIによって固形がんのMRイメージングを行った結果を図4fに示す。この結果、Gd-DTPAの投与ではMRIシグナルの増大は確認されなかったが、Gd-DTPA内包ミセルの投与によって腫瘍特異的に信号強度が増大することが確認された。

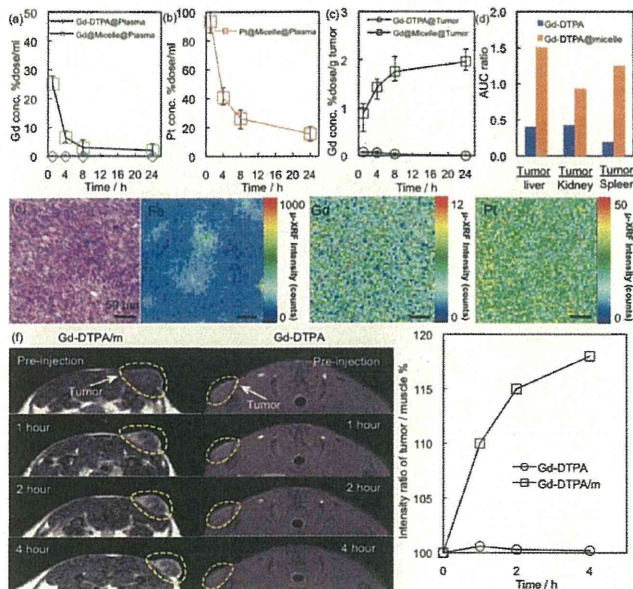


図4. (a-d) C26細胞の皮下移植モデルマウスにおけるGdおよびPtの血中滞留性、Gdの固形がんへの集積性、正常臓器とがんのGdの集積比。(e)  $\mu$ -SR-XRFによるFe、Gd、Ptの元素マッピング。(f) 固形がんのMRイメージングの結果

#### D. 考察

本研究では、Ptをリンカーとして可逆的な配位結合によりGd-DTPAをポリマーに結合した高分子ミセルを開発した。ポリマーにDTPAやDOTA等のキレート化剤を共有結合した場合には、Gd-DTPAがリリースされないために長期にわたり体内に滞留することから起因する毒性の高いGd<sup>2+</sup>イオンのリリースや肝臓等へのGdの蓄積毒性が問題となるが、本システムでは生理的条件下において10時間で約80%のGd-DTPAをリリースすることが確認されており(Cabralの分担報告書参照)、リリースされたGd-DTPAは速やかに腎糸球体よりろ過排泄されるものと考えられることから体内への蓄積毒性を示さないものと考えられる。

一方、本研究ではこれまでに、DachPtとGd-DTPAを同時に搭載した診断-治療機能一体型DDSの開発を行ってきたが、本システムは投与量に関して、制がん剤の最大耐用量に基づく制限があり、鮮明なイメージングを得るために投与量を上げることが困難であった。このため本研究では、制がん活性を持た

ないH<sub>2</sub>PtCl<sub>6</sub>と生分解性を有することにより蓄積毒性を示さないPEG-PAsp (DET)の金属錯体形成により高分子ミセルを形成させ、Ptの可逆的な金属錯体形成を介してGd-DTPAを内包させた系を開発した。そこで本システムの毒性を評価するために、HUVECに対する細胞毒性を評価したところ、Gd-DTPA内包ミセルならびにそれらの構成成分はHUVECに対して細胞毒性を示さないことが明らかになった(図2)。この結果より、Gd-DTPA内包ミセルは、DACHPt/Gd-DTPA内包ミセルよりも毒性が低く、投与量が高めることが可能となった(data not shown)。

次に、Gd-DTPA内包ミセルのT1緩和能をパルスNMRを用いたファントム実験により評価した(図2)。その結果、Gd-DTPA内包ミセルは、Gd-DTPAの13.1倍のT1緩和能を有することが明らかとなった。この結果は、ミセル内部で、Gdの分子内運動が抑制( $\tau_R$ 値が増加)され、Gdとプロトンの接触機会が増加ことに加え、水分子が次々に交換できる環境(低い $\tau_m$ 値)が実現されていることに起因するものと考えられる。すなわち、Gd-DTPA内包ミセルはT1造影剤として優れた性能を有することが明らかになった。

最後に、Gd-DTPA内包ミセルの体内動態評価と固形がんモデルのMRイメージングを行った(図4)。その結果、Gd-DTPAがリリースされるために、Gdの血中濃度はPtのそれより低くなるものの、Gd-DTPA単独よりは高い血中滞留性を示し、結果的に固形がんにも効果的かつ選択的に集積することが示された。この結果は、10時間で約80%のGd-DTPAをリリースするというGd-DTPA内包ミセルの特性が、EPR効果によって固形がんへのGd-DTPAの集積を高めつつ、糸球体ろ過によってGd-DTPAを体外に排泄させるために理想的なものであることを示唆しているものと考えられる。この特性によって、Gd-DTPA内包ミセルは、腫瘍特異的にMRIの信号強度が増大させることが明らかになった。

以上の検討より、本年度は、制がん活性を持たないGd-DTPA内包ミセルを開発し、その基本性能を明らかにすることができた。本システムは、Gd-DTPAのがん集積性を高める一方で、ミセルからGd-DTPAをリリースすることによって蓄積毒性を示さないシステムとしてがんの精密診断用ナノデバイスとしての利用が期待される。

#### E. 結論

前年度までに、Gd-DTPAを搭載し、DachPtによる治療効果をMRIによって追跡できる診断-治療機能一体型DDSの開発を行い、Theranostic Nanodeviceのコンセプトを実証することができた。しかしながら、本システムは投与量に関して、制がん剤の最大耐用量に基づく制限があり、鮮明な固形がんイメージングを目的として投与量を上げるためには、制がん活性を持たないGd-DTPA内包ミセルを開発する必

要があるものと考えられた。そこで3年計画の最終年度にあたる本年度は、制がん活性を持たないGd-DTPA内包ミセルを開発し、MRI診断用ナノデバイスとしての有用性を明らかにすることができた。本システムは、Gd-DTPAのがん集積性を高める一方で、ミセルからGd-DTPAをリリースすることによって蓄積毒性を示さないシステムとしてがんの精密診断用ナノデバイスとしての利用が期待される。

#### F. 健康危険情報

本研究では現在のところ健康に危険を及ぼす可能性はない。

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## 2. 学会発表

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H. 知的財産権の出願・登録状況  
なし



厚生労働科学研究費補助金（医療機器開発推進研究事業）  
分担研究報告書

固形がんの標的治療とその治療効果のMRIによる追跡を可能にする  
診断-治療機能一体型DDSの創製  
(制がん活性を持たないGd-DTPA内包ミセルの調製と物性評価)

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特任講師

研究要旨

本研究では、固形がんの精密診断に有用な診断用ナノデバイスとして、制がん活性を持たないヘキサクロロ白金酸の金属錯体形成を利用することによってMRI用造影剤Gd-DTPAを内包させた高分子ミセルを開発した。開発したGd-DTPA内包ミセルに関して、サイズ、形態(morphology)、生理的条件下における安定性等の基本物性を詳細に検討し、明らかにした。

A. 研究目的

MRIは、がんの非侵襲的診断法として極めて重要であるが、精度向上の為にMRI造影剤の開発が重要である。これまでにGd-DTPA(マグネビスト)および磁性微粒子がそれぞれ脳腫瘍および肝がんの画像診断に利用されているが、前者では脳腫瘍で血液-脳関門が一部破綻していること、後者では微粒子が肝臓のクッパー細胞に貪食されることを利用しており、上記以外の固形がんのMRI診断に応用することが困難である。従って、がん組織に選択的に集積し、がんのMRI診断に広く利用できるMRI造影剤が望まれているが、そのような造影剤は未だ開発されていないのが現状である。

本研究では、前年度までに制がん剤による治療効果をMRIによって追跡できる診断用ナノデバイスとして、DACHPt/Gd-DTPA内包ミセルを開発し、マウス腫瘍がんモデルにおいてその有用性を明らかにしてきた。しかしながら、本システムは投与量に関して、抗がん剤の最大耐用量に基づく制限があり、鮮明な固形がんイメージングを目的として投与量上げるためには、制がん活性を持たないGd-DTPA内包ミセルを開発する必要があるものと考えられた。そこで本年度は、制がん活性を持たないヘキサクロロ白金酸と生分解性を有することにより蓄積毒性を示さないPEG-PAsp(DET)の金属錯体形成により高分子ミセルを形成させ、Ptの可逆的な金属錯体形成を介してMRI用陽性造影剤のGd-DTPAを内包させた系を開発した(図1)。さらに、開発したGd-DTPA内包ミセルに関して、サイズ、形態(morphology)、生理的条件下における安定性等の基本物性を詳細に検討した。

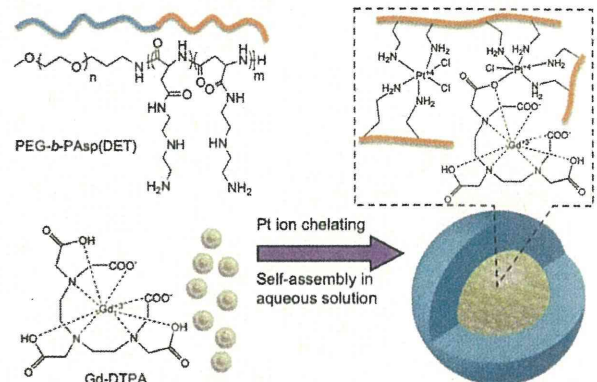


図1. 白金の金属錯体形成を利用してGd-DTPAを内包した高分子ミセル

B. 研究方法

1. Gd-DTPA内包ミセルの調製

Poly(ethylene glycol)-*b*-poly(*N*-(*N*'-(2-aminoethyl)-2-aminoethyl aspartamide)) (PEG-*b*-P(Asp(DET))) (P(Asp(DET))の重合度: 45)とH<sub>2</sub>PtCl<sub>6</sub>、Gd-DTPAを水中で混合し、120時間反応させることによって、Gd-DTPA内包ミセルを調製した。ここで、[Gd-DTPA]/[DET] = 1.0として、Pt:Gd比を変化させてミセル調製を行い、動的光散乱(DLS)測定によってミセル形成の有無を確認した。また、ICP-MSを利用してPtおよびGd-DTPAの導入率を算出し、ミセル形成のための最適条件を検討した。

上記の検討によって最適化された高分子ミセルに関して、透過型電子顕微鏡(JEM-1400, JEOL, Japan)を用いた形態(morphology)の評価を行った。さらにScanning transmission electron microscopy

(STEM)観察を行い、元素マッピングを行うことによって、PtとGdの分布を評価した。

## 2. Gd-DTPA内包ミセルの安定性評価

生理的条件下(150mM NaCl、pH7.4、37°C)におけるGd-DTPA内包ミセルの安定性とPtおよびGdのリリースを評価した。ここで、ミセルの安定性は、DLSによって、散乱光強度と粒径の変化を測定することによって評価し、ミセルからのPtおよびGdのリリース、ミセル溶液を透析した後の外液中のPtおよびGdの量をICP-MSで定量することによって評価した。

### C. 研究結果

#### 1. Gd-DTPA内包ミセルの調製

仕込みにおける[Pt]/[Gd-DTPA]のモル比を変化させてミセルを調製し、形成されたミセルのHydrodynamic diameterとpolydispersity index(PDI)をDLSによって評価した(図2 a-b)。その結果、[Pt]/[Gd-DTPA]比が5および10の時に、粒径が40-50nmでPDIが0.1以下の水中で安定な高分子ミセルが形成された。また、Gdの内包率を検討したところ、[Pt]/[Gd-DTPA]比が5および10の時に、内包率がそれぞれ16%および12%であったことから、以降の実験では[Pt]/[Gd-DTPA]=5の条件でミセル調製を行った。この条件で形成されたミセルは非常に狭い粒径分布を有することが確認された(図2 c)。

次に、[Pt]/[Gd-DTPA]=5の条件で形成されたGd-DTPA内包ミセルのTEM観察を行った(図2 d-f)。その結果、粒径22nmの単分散な粒子の形成が確認された。DLS測定で数平均分布におけるGd-DTPA内包ミセルの粒径が23nmであったことから、TEMとDLSで矛盾しない結果が得られたものと思われる。

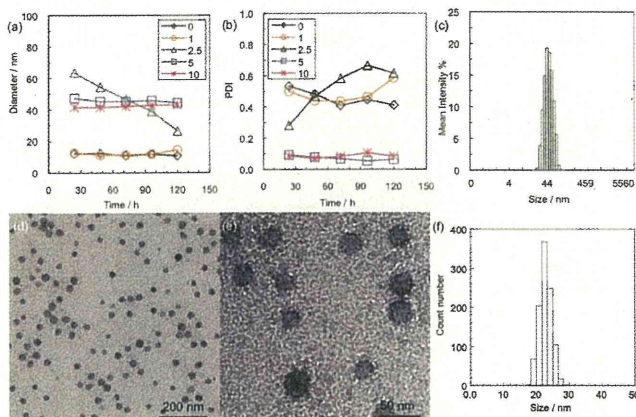


図2. (a-c) [Pt]/[Gd-DTPA]比を変化させた時の高分子ミセルDLS測定の結果、(d-f) Gd-DTPA内包ミセルのTEM測定の結果

さらに、Gd-DTPA内包ミセルのSTEM観察の結果を図3に示す。元素マッピングの結果、粒子内にPtとGdが存在していることが確認された(図3 b,

c)。

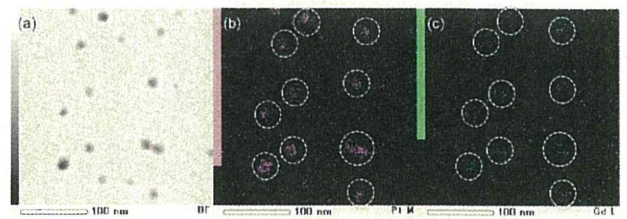


図3. (a) Gd-DTPA内包ミセルのSTEM測定の結果、(b) Ptのマッピング、(c) Gdのマッピング

#### 2. Gd-DTPA内包ミセルの安定性評価

Gd-DTPA内包ミセルの生理的条件下(150mM NaCl、pH7.4、37°C)における安定性をDLS測定によって評価した(図4 a,b)。その結果、48時間経過後もミセルの粒径に変化は見られないが、散乱光強度は時間依存的にゆっくりと減少し、初期の60%程度に達することが確認された。さらに、PtおよびGdのリリースを評価したところ、Ptはほとんどリリースされないが、Gdは10時間をかけてゆっくりとリリースされ、内包量の80%まで到達することが確認された(図4 c)。これらの結果は、Gd-DTPAを徐放するシステムであることを示唆している。

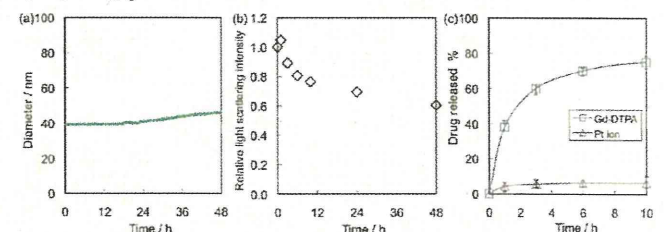


図4. 生理的条件下(150mM NaCl、pH7.4、37°C)におけるGd-DTPA内包ミセルの安定性評価[(a) 粒径変化、(b) 散乱光強度変化、(c) PtおよびGdのミセルからのリリース]

### D. 考察

これまでに、ポリマーにDTPAやDOTA等のキレート化剤を共有結合し、Gd<sup>2+</sup>イオンを搭載したナノデバイスは報告されているが、この場合、Gd-DTPAがリリースされないために長期にわたり体内に滞留することに起因する毒性の高いGd<sup>2+</sup>イオンのリリースや肝臓等へのGdの蓄積毒性が問題となる。そこで本研究では、Ptをリンカーとして可逆的な配位結合によりGd-DTPAをポリマーに結合した高分子ミセルを開発した。[Pt]/[Gd-DTPA]のモル比を最適化した結果、40-50nmの非常に単分散なGd-DTPA内包ミセルが形成されることが確認された。ICP-MSによってGd-DTPA導入率を評価した結果、16%と比較的高い内包率を達成することができた。さらに、生理的条件下(150mM NaCl、pH7.4、37°C)におけるGd-DTPA内包ミセルの安定性を評価した結果、Ptはミセルからほとんどリリースされないが、Gd-DTPAは10

時間で約80%がリリースされることが確認された。ミセルはEPR効果によって数時間でがん組織に集積し、24時間後に血中から消失することを考えると、このリリースのプロファイルはGd-DTPAのがんへの集積を達成しつつ、糸球体ろ過によってGd-DTPAを体外に排泄させるために理想的なものであると考えられる。このGd-DTPA内包ミセルのMRI造影剤としての性能評価は研究代表者の西山によって実施されており、詳細は総括研究報告書に記載した。

#### E. 結論

本分担研究では、Gd<sup>2+</sup>イオンの金属錯体形成を介して、共有結合を使用せずに、ポリマーにGd-DTPAを内包させた高分子ミセルを開発した。Gd-DTPA内包ミセルは、45nmの単分散な粒径を有しており、水中では非常に安定であるが、生理的条件下においては、10時間で約80%のGd-DTPAをリリースすることが確認された。本システムは、Gd-DTPAのがんへの集積を達成しつつ、糸球体ろ過によってGd-DTPAを体外に排泄させることのできるMRI診断用ナノデバイスとして今後の展開が期待される。

#### F. 健康危険情報

本研究では現在のところ健康に危険を及ぼす可能性はない。

#### G. 研究発表

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H. 知的財産権の出願・登録状況  
なし

## 研究成果の刊行に関する一覧表

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## Polymeric micelles incorporating (1,2-diaminocyclohexane)platinum (II) suppress the growth of orthotopic scirrhous gastric tumors and their lymph node metastasis

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### ABSTRACT

Nano-scaled drug carriers have great potential for the treatment of solid tumors. Nevertheless, hypovascularity and fibrosis in some types of solid tumors have been demonstrated to reduce the penetration and accumulation of nano-scaled drug carriers. Diffuse-type scirrhous gastric cancers present such characteristics as well as frequent metastasis to the lymph nodes; therefore, it remains a great challenge to eradicate scirrhous gastric cancers based on the drug targeting using nanocarriers. Herein, we demonstrated that polymeric micelles with 30-nm diameter incorporating (1,2-diaminocyclohexane)platinum(II) (DACHPt), the parent complex of the anticancer drug oxaliplatin, efficiently penetrated and accumulated in an orthotopic scirrhous gastric cancer model, leading to the inhibition of the tumor growth. Moreover, the elevated localization of systemically injected DACHPt-loaded micelles in metastatic lymph nodes reduced the metastatic tumor growth. These results suggest DACHPt-loaded micelles as a promising nanocarrier for the treatment of scirrhous gastric cancers and their lymphatic metastases.

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### 1. Introduction

Nano-scaled drug carriers are being developed for improving the treatment of solid tumors while decreasing the toxicity [1–5]. These nanocarriers effectively accumulate in solid tumors due to the hyper-permeability of blood capillaries to circulating macromolecules and the impaired lymphatic drainage of these macromolecules, which are known as the Enhanced Permeability and Retention (EPR) effect [6]. Indeed, several nanocarrier formulations have been approved for clinical use against hypervascular cancers such as ovarian cancers, HIV-associated Kaposi's sarcoma and breast cancers [7,8]. However, in some intractable cancers such as pancreatic and gastric cancers, the hypovascularity and fibrosis of tumors may compromise the extravasation and tissue penetration of nanocarriers [9,10].

Gastric cancers cause 1 million deaths per year worldwide being the 2nd leading cause of cancer-related death following lung cancer [11]. Among gastric cancers, a diffuse-type scirrhous gastric cancer (SGC) affects younger patients and presents the highest mortality [12]. SGC is characterized by hypovascularity, extensive stromal fibrosis and metastasis to the lymph nodes [13–15]. These characteristics impair the therapeutic efficacy of chemotherapy as well as nanocarrier-mediated targeting chemotherapy. Moreover, the targeting chemotherapy against lymph node metastasis involve intralymphatic or local administration of nanocarrier-encapsulated antitumor agents [16–18]; however, these approaches may not target all draining lymph nodes due to the inappropriate position of the injection [18] and the obstruction of lymphatic vessels in advanced stages of cancer [18–19]. Hence, the improved targeting against the lymph node metastasis is strongly needed to eradicate the lymph node metastasis.

Polymeric micelles, self-assemblies of block copolymers are characterized by the core-shell structures with drug-loaded core surrounded by hydrophilic PEG shell, and have shown great potential as tumor-targetable nanocarriers [3–5]. The substantial advantages of polymeric micelles include relatively small size ranging from 20 to 100 nm, controllable drug loading and release, and favorable bio-distribution including prolonged blood circulation and enhanced tumor accumulation [3–5]. Accordingly, micelle formulations

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incorporating doxorubicin, SN-38, paclitaxel, cisplatin, and (1,2-diaminocyclohexane)platinum(II) (DACHPt) (an active form of oxaliplatin) exerted significant efficacy against several tumor models with appreciably lowered toxicity compared to free drugs, and are currently under clinical evaluation [20–24]. Particularly, DACHPt-loaded polymeric micelles (DACHPt/m) are characterized by the small size (ca. 30 nm) [25–26], achieving high penetration into tumor mass and remarkable antitumor activity against poorly permeable tumors such as pancreatic tumors [27–29].

This study was aimed to evaluate the targeting ability and therapeutic efficiency of systemically injected DACHPt/m against a well-established experimental model of SGC, which was prepared by orthotopic inoculation of OCUM-2MLN scirrhous gastric cancer cells [30–31]. In the orthotopic SGC model of OCUM-2MLN cells, the induction of lymph node metastasis occurs in all mice, while the tumor microenvironment shows hypovascularity and thick fibrosis similar to SGC in the patients, indicating the clinical relevancy of this model [30–31]. Here, the therapeutic potential of the DACHPt/m against orthotopic SGC and their lymph node metastasis was examined by using bioluminescent OCUM-2MLN-Luc tumors.

## 2. Materials and methods

### 2.1. Materials

Bis(trichloromethyl)carbonate (triphosgene) was purchased from Tokyo Kasei Kogyo Co., Inc. (Tokyo, Japan).  $\gamma$ -Benzyl L-glutamate was purchased from Sigma Chemical Co., Inc. (St. Louis, MO). *N,N*-Dimethylformamide (DMF) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Wako Pure Chemical Co., Inc. (Osaka, Japan). Dichloro(1,2-diaminocyclohexane)platinum(II) (DACHPtCl<sub>2</sub>) was purchased from Heraeus (Germany). AgNO<sub>3</sub> was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).  $\alpha$ -Methoxy- $\omega$ -amino poly(ethylene glycol) (CH<sub>3</sub>O-PEG-NH<sub>2</sub>; Mw = 12,000) was purchased from NOF Co., Inc. (Tokyo, Japan). Alexa 594- and Alexa 680-N-hydroxysuccinimide ester were purchased from Invitrogen (USA).

### 2.2. Animals and cells

Immunodeficient BALB/c nu/nu mice at 6 weeks of age were obtained from Charles River Laboratories (Tokyo, Japan), and treated in accordance with the policies of the Animal Ethics Committee of The University of Tokyo. Human diffuse-type gastric carcinoma OCUM-2MLN cells were used for the model of diffuse-type scirrhous gastric cancer (SGC) with high frequency of the lymph node metastasis [30–31]. OCUM-2MLN cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Sigma Chemicals) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C.

### 2.3. Preparation of PEG-*b*-P(Glu)

Poly(ethylene glycol)-poly(L-glutamic acid) block copolymers (PEG-*b*-P(Glu)) were synthesized according to the previously described synthetic method with a slight modification [25]. Briefly, *N*-carboxy anhydride of  $\gamma$ -benzyl L-glutamate was polymerized in DMF, initiated by the primary amino group of CH<sub>3</sub>O-PEG-NH<sub>2</sub> to obtain PEG-poly( $\gamma$ -benzyl-L-glutamate) block copolymer (PEG-*b*-PBLG). A narrow distribution (Mw/Mn: 1.06) of PEG-*b*-PBLG was confirmed by gel permeation chromatography [column: TSK-gel G3000HHR, G4000HHR (Tosoh, Yamaguchi, Japan); eluent: DMF containing 10 mM LiCl; flow rate: 0.8 ml/min; detector: refractive index (RI); temperature: 25 °C]. The polymerization degree of PBLG was determined to be 20 by comparing the proton ratios of methylene units in PEG (—OCH<sub>2</sub>CH<sub>2</sub>—:  $\delta$  = 3.7 ppm) and phenyl groups of PBLG (—CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>—:  $\delta$  = 7.3 ppm)

in <sup>1</sup>H-NMR measurement [JEOL EX270 (JEOL, Tokyo, Japan); solvent: DMSO-*d*<sub>6</sub>; temperature: 80 °C]. The deprotection of the benzyl group of PEG-*b*-PBLG was carried out by mixing with 0.5 N NaOH at room temperature to obtain PEG-*b*-P(Glu).

PEG-*b*-P(Glu) was fluorescently labeled by the conjugation of Alexa 594- or Alexa 680-succinimidyl esters to the  $\omega$ -amino group of the polymer in DMSO. Twenty-four hours later, the polymers were purified by dialysis against DMSO followed by water. Finally, the solutions were freeze-dried, and the amount of conjugated dye was determined to be approximately 70% (% fluorescent dye / PEG-*b*-P(Glu)) by fluorescence spectroscopy.

### 2.4. Preparation of DACHPt-loaded micelles (DACHPt/m)

DACHPt/m were prepared according to a previously described method [25]. Briefly, DACHPtCl<sub>2</sub> (5 mM) was suspended in distilled water and mixed with silver nitrate [(AgNO<sub>3</sub>)/(DACHPt) = 1] to form DACHPtCl(NO<sub>3</sub>). The solution was kept in the dark for 24 h at 25 °C. AgCl precipitate after reaction was eliminated by centrifugation. The supernatant was purified by passage through a 0.22  $\mu$ m filter. Then, PEG-*b*-P(Glu) [(Glu) = 5 mmol/l] was added to the DACHPt solution [(DACHPt)/(Glu) = 1.0] and reacted for 120 h to prepare DACHPt/m. The prepared micelles were purified by ultrafiltration [molecular weight cutoff size (MWCO): 30,000]. The size distribution of DACHPt/m was evaluated by dynamic light scattering (DLS) measurement at 25 °C, using the Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, United Kingdom).

The Pt content of the micelles was determined by using an ion coupled plasma-mass spectrometer (4500 ICP-MS; Hewlett Packard, Palo Alto, CA). Fluorescent-labeled DACHPt/m were prepared in a similar manner with Alexa 594- or Alexa 680-labeled PEG-*b*-P(Glu).

#### 2.4.1. *In vitro* cytotoxicity assay

To determine the cytotoxicity, OCUM-2MLN cells were seeded in a 96 well plate and incubated at 37 °C. Then, the cells were exposed to free oxaliplatin or DACHPt/m for 48 or 72 h, followed by the addition of MTT solution. Cell viability was measured by the formed formazan absorbance at 570 nm.

### 2.5. Preparation of orthotopic SGC models with lymphatic metastasis

Lentiviral vector [32] was used to express the luciferase protein gene stably in OCUM-2MLN cells. The prepared OCUM-2MLN-Luc cells were grown in DMEM containing 10% FBS. For preparation of orthotopic SGC models, OCUM-2MLN-Luc cells were inoculated by orthotopic implantation method. Briefly, after the abdomen of BALB/c nu/nu mice was sterilized with alcohol, an incision was made to expose the stomach, and OCUM-2MLN-Luc cell suspension was injected sub-serosally into the gastric walls as previously described [33]. The stomach was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with surgical suture.

### 2.6. Evaluation of antitumor activity by bioluminescent imaging

Bioluminescent imaging [34] was carried out with a highly sensitive CCD camera using IVIS imaging system (IVIS, Caliper Life Science, Hopkinton, MA). Imaging and quantification of signals were analyzed by Living Image software (Caliper Life Science). For *in vitro* imaging, bioluminescent cells were diluted in cell culture media in a black, 96-well plate (Costar, Corning, NY). VivoGlo luciferin (Promega, Madison, WI) at 150  $\mu$ g/ml in media was added to each well 5 min prior to imaging. Imaging was performed for 1 min per plate. For the analysis of antitumor activity, BALB/c nu/nu ( $n$  = 5) mice were inoculated by orthotopic injection of OCUM-2MLN-Luc cells ( $5 \times 10^6$ ). Tumors were allowed to grow for 3 weeks, to initiate the metastasis to the lymph nodes. Subsequently, the mice received three *i.v.* injections of oxaliplatin (8 mg/kg)

and DACHPt/m (4 mg/kg on a Pt base) on days 0, 2 and 4. Photon flux was measured until 2 weeks and was taken as a parameter of tumor volume. Body weight was measured simultaneously to assess the toxicity.

### 2.7. Antitumor activity against lymph node metastasis

To study the antitumor efficacy of the DACHPt/m against lymphatic metastasis, the mice were sacrificed at the end of the antitumor activity experiment and the metastatic lymph nodes were excised and weighed. The inhibition of the growth of the metastases was associated with the weight of the lymph nodes.

### 2.8. Accumulation of DACHPt/m in orthotopic tumors and metastatic lymph nodes

In order to analyze the biodistribution of oxaliplatin and DACHPt/m, OCUM-2MLN-Luc cells ( $5 \times 10^6$  cells/ml) were injected sub-serosally into the gastric walls of BALB/c nu/nu mice (female,  $n = 5$ ). Four weeks later, oxaliplatin and DACHPt/m were intravenously injected at a dose of 100  $\mu\text{g}$ /mouse on a platinum basis. Mice were sacrificed after defined time periods (1, 4 and 24 h), and then tumor and metastatic lymph nodes were collected. Tissue samples were washed in ice-cold saline and weighed after removing excess fluid. All samples were dissolved in  $\text{HNO}_3$  and evaporated to dryness. The Pt concentration was measured by ICP-MS after the samples were redissolved in 5N HCl.

### 2.9. Biodistribution of fluorescent-labeled DACHPt/m

DACHPt/m were labeled with Alexa 680 to investigate the accumulation in orthotopic gastric tumors and metastatic lymph nodes. OCUM-2MLN ( $5 \times 10^6$  cells/ml) cells were inoculated sub-serosally into the gastric walls of BALB/c nu/nu mice and the tumors were allowed to grow for about 3 weeks. Alexa 680-labeled DACHPt/m (5 mg/kg on Pt basis) were intravenously injected and the mice were imaged after 24 h to track the accumulation of micelles in the

whole body by using IVIS imaging system (excitation: 640 nm; emission: 720 nm; exposure time: 1 s; field of view: 18.1 cm). Then, the mice were sacrificed and the organs (liver, kidney and spleen), the primary tumor and the metastatic lymph nodes were imaged *ex vivo* to evaluate the accumulation of micelles.

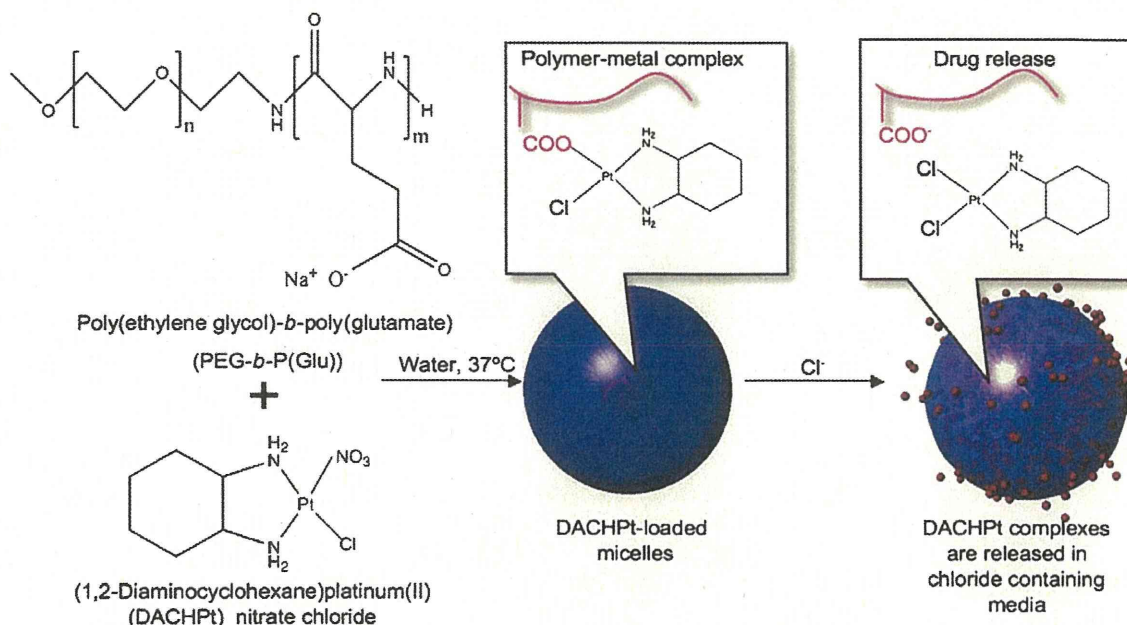
### 2.10. Microdistribution of fluorescent micelles in orthotopic tumor and lymph node metastasis

Tumor bearing mice were injected intravenously with Alexa 594-labeled DACHPt/m to evaluate their microdistribution in primary tumors and metastatic lymph nodes by histology. Tumors, metastatic and normal lymph nodes were excised, snapped frozen in liquid nitrogen and embedded in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek). The frozen samples were sectioned at 10- $\mu\text{m}$  thickness in a cryostat and fixed with 10% formalin. The tissue sections were stained with hematoxylin and eosin (H&E) and observed using an Olympus AX80 microscope (Olympus, Japan). For immunohistochemical analysis, the tissue sections were also stained for lymphatic vessels by using anti-LYVE-1 antibody (Abcam), blood vessels by using anti-PECAM-1 antibody (BD PharMingen), and cancer cells by using anti-CD326 antibody (EpCAM, Miltenyi biotec), followed by staining with Alexa488- and Alexa647-conjugated secondary antibodies (Invitrogen). Hoechst 33342 was used for nuclear staining. The stained tissue samples were observed using a LSM510 META laser confocal microscope (Carl Zeiss, Germany).

## 3. Results

### 3.1. In vitro characterization of DACHPt-loaded micelles (DACHPt/m)

The polymer-metal complex formation between DACHPt and the carboxylic group of the P(Glu) in the PEG-*b*-P(Glu) led to the formation of polymeric micelles with an average diameters of approximately 30-nm (Fig. 1 and Supplementary Fig. S1A). DACHPt/m can release cytotoxic DACHPt complexes from the micelle core by the ligand



**Fig. 1.** Scheme of the preparation of DACHPt-loaded micelles (DACHPt/m). The micelles are formed by the polymer-metal complex formation between DACHPt and the carboxylic acid moieties in the poly(glutamic acid) backbone. DACHPt is released in a sustained manner as a result of the ligand substitution of the Pt(II) from the carboxylates in the core of the micelles with the chloride ions in the medium.



**Table 1**  
Fifty-percent cell growth inhibitory concentration ( $IC_{50}$ ) of DACHPt/m and oxaliplatin against human scirrhous gastric cancer OCUM-2-MLN cells.

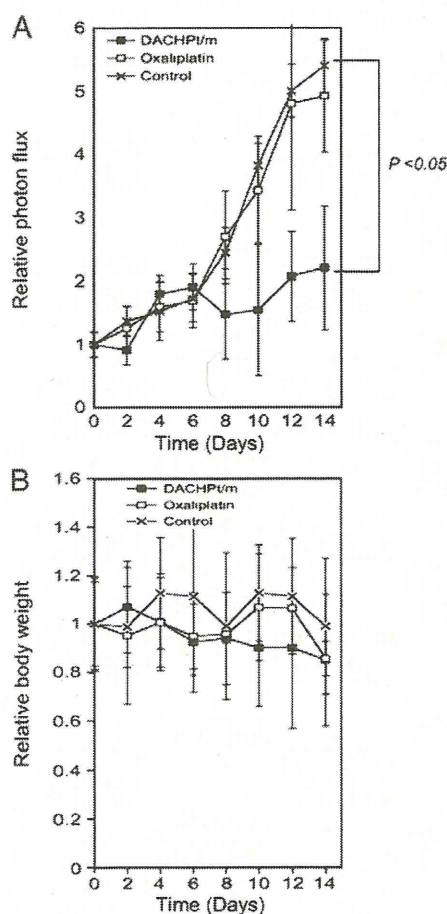
Drug	$IC_{50}$ ( $\mu$ M)	
	48 h	72 h
DACHPt/m	20.0	12.5
Oxaliplatin	12.5	6.25

exchange of DACHPt from the carboxylic groups of p(Glu) to the chloride ions in the media [28]. The 50% cell growth inhibitory concentration ( $IC_{50}$ ) of DACHPt/m was approximately 2-fold higher than that of oxaliplatin (Table 1).

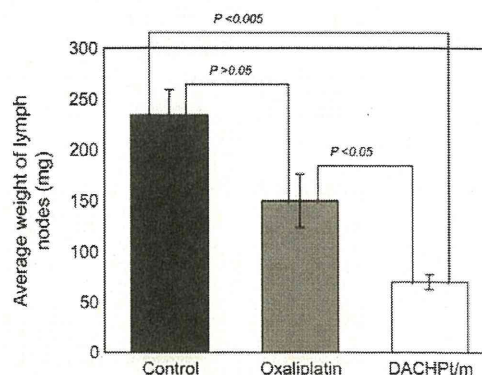
### 3.2. Antitumor activity against bioluminescent orthotopic gastric tumors

OCUM-2MLN cells stably expressing luciferase gene (OCUM-2MLN-Luc) were prepared by the lentiviral transfection. The bioluminescent signal of OCUM-2MLN-Luc after the addition of luciferin was measured by IVIS (Supplementary Fig. S2). As the light glowing from the cells increased linearly with cell density (Supplementary Fig. S2), we can use the light intensity of OCUM-2MLN-Luc to quantify the tumor burden.

The antitumor activity of oxaliplatin and DACHPt/m was studied in mice bearing orthotopic OCUM-2MLN-Luc tumors that have



**Fig. 2.** Antitumor activity of oxaliplatin and DACHPt/m against bioluminescent OCUM-2-MLN-Luc orthotopic gastric cancer. A, Relative photon flux from the tumors after the injection of saline, oxaliplatin 8 mg/kg and DACHPt/m 4 mg/kg on days 0, 2 and 4. B, Relative body weight of the mice. Data are expressed as a mean  $\pm$  SE ( $n=5$ ).



**Fig. 3.** Weight of the metastatic lymph nodes at the end of the antitumor activity experiment (day 14). Data are expressed as a mean  $\pm$  SE ( $n=5$ ).

already developed metastasis to the lymph nodes. The mice were treated intravenously 3 times at 0, 2 and 4 days with oxaliplatin at 8 mg/kg or DACHPt/m at 4 mg/kg on a Pt basis. The photon flux (photons per second) from the bioluminescent tumors was assessed by IVIS every second day. Consequently, oxaliplatin failed to show any antitumor effect against the OCUM-2MLN-Luc tumors as the photon flux of oxaliplatin-treated mice was comparable to that of untreated mice (Fig. 2A). In contrast, the mice treated with DACHPt/m showed significantly lower relative photon flux compared with untreated and oxaliplatin-treated mice (Fig. 2A) without significant body weight loss (Fig. 2B), indicating a significant antitumor activity against the orthotopic gastric tumors.

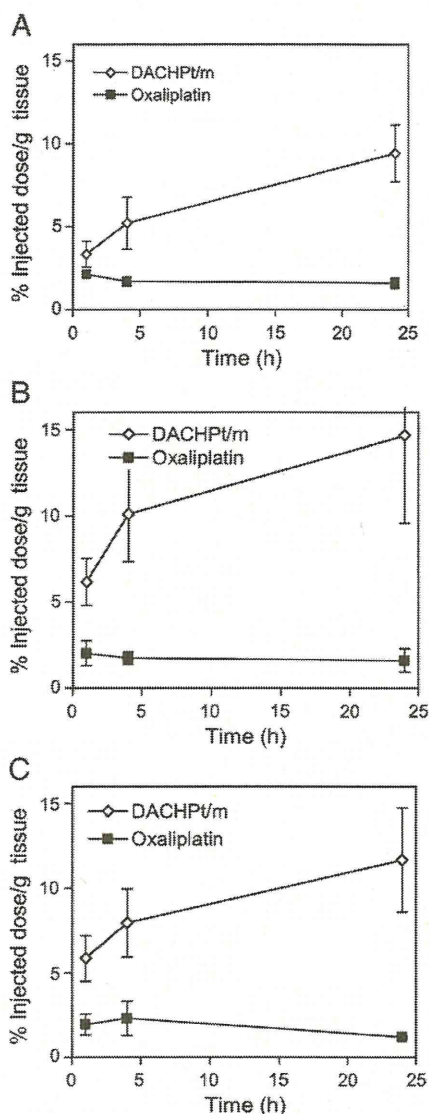
### 3.3. Growth inhibition of lymph node metastasis

At the end of the antitumor activity study, the mice were sacrificed and the metastatic lymph nodes were collected and weighed. As shown in Fig. 3, the weights of the lymph nodes in DACHPt/m-treated mice were approximately four and two times lower than those in untreated mice and oxaliplatin-treated mice, respectively, suggesting that DACHPt/m might effectively inhibit the growth of the metastatic lymph nodes and their efficacy might surpass that of oxaliplatin.

### 3.4. Biodistribution of DACHPt/m in orthotopic gastric tumors and metastatic lymph nodes

DACHPt/m gradually accumulated in the orthotopic tumors due to the EPR effect, achieving approximately 10% of injected dose per gram of tumor tissue at 24 h (Fig. 4A). The tumor accumulation at 24 h for DACHPt/m was 6 times higher than oxaliplatin (Fig. 4A). DACHPt/m also showed enhanced accumulation in sentinel and distant metastatic lymph nodes (Fig. 4B and C). The effective accumulation of DACHPt/m in the metastatic lymph nodes may account for their effective growth inhibition of lymphatic metastasis.

The *in vivo* distribution of fluorescent-labeled DACHPt/m was assessed by fluorescent imaging of the whole body of tumor-bearing mice. Twenty-four hours after intravenous injection of fluorescent-labeled DACHPt/m, intense fluorescence signal was detected at the tumor site, suggesting the selective accumulation of DACHPt/m in the orthotopic tumors (Fig. 5A). In addition, mice were sacrificed and the tumor, metastatic lymph nodes and organs (i.e., spleen, liver and kidneys) were imaged *ex vivo* (Fig. 5B). The high fluorescence signal of the micelles in the tumor and metastatic lymph nodes indicates the ability of the micelles to target the scirrhous gastric tumors and their lymphatic metastases.



**Fig. 4.** Accumulation of oxaliplatin and DACHPt/m in OCUM-2-MLN-Luc orthotopic tumors and lymph node metastasis. A, Orthotopic tumor. B, Primary metastatic lymph node (sentinel lymph node). C, Secondary metastatic lymph node. Data are expressed as a mean  $\pm$  SE ( $n=5$ ).

### 3.5. Microdistribution of fluorescent-labeled DACHPt/m in orthotopic tumors and metastatic lymph nodes

Firstly, the histology of OCUM-2MLN tumors was examined by H&E staining (Fig. 6A). These tumors present poorly differentiated characteristics with hypovascularity and extensive stromal fibrosis of gastric cancers [9]. The microdistribution of the fluorescent-labeled DACHPt/m in the tissue sections of the orthotopic gastric tumors was assessed by fluorescence microscopy. The nucleus of the cells in the whole tissue sections were stained with Hoechst (Fig. 6B, blue) while the tumors regions were stained by using anti-CD326 antibody (Fig. 6B, green), which recognizes the human epithelial antigen (HEA). The colocalization of the fluorescent-labeled DACHPt/m (Fig. 6B, magenta) with the CD326-stained cancer cells (Fig. 6B, green) suggests the homogeneous accumulation of the micelles in the tumor tissue (Fig. 6B). Moreover, the blood vessels,

marked with anti-PECAM-1 antibody, were not detectable within the tumor (Fig. 6C, green), suggesting the hypovascular nature of the tumors. Also, the lymphatic vessels, marked with anti-LIVE-1 antibody, were not detected inside the tumor tissue (Fig. 6C, blue). In OCUM-2MLN tumor model, the cancer cells exhibit tumor invasion into peritumoral lymphatic vessels and spread along the lymphatic vessels in the gastric wall to the regional lymph nodes [33]. The fluorescence of the micelles was detected in the tumor sections even at the regions distant from the blood vessels (Fig. 6C, magenta), suggesting the deep penetration of DACHPt/m within the tumor tissue.

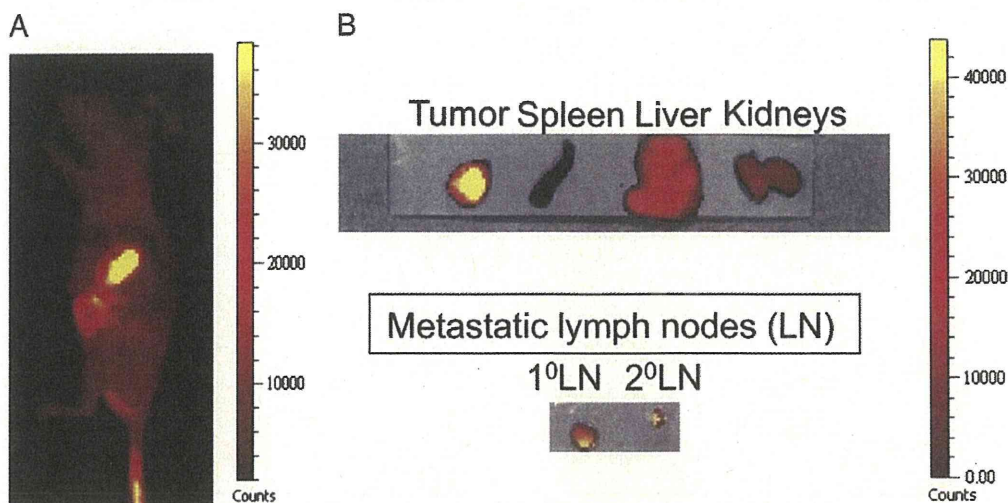
The H&E staining of the metastatic lymph nodes (Fig. 7A) indicated the abnormal anatomy of the lymph node due to the tumor growth. Fluorescent signal from the micelles was found to be colocalized with cancer cells, stained by CD326, in the metastatic lymph nodes, suggesting that micelles deeply penetrate into the metastases in the lymph nodes (Fig. 7B). Additionally, the lymphatic tissue and blood vessels were stained with anti-LYVE-1 and anti-PECAM-1 antibodies, respectively (Fig. 7C). LYVE-1-positive lymphatic tissue was mainly found in the periphery of the tumor in the lymph node (Fig. 7C, blue). Fluorescent-labeled micelles were found to accumulate within the tumoral region of the metastatic lymph nodes (Fig. 7C, magenta).

It is assumed that polymeric micelles from the circulation may reach the lymph nodes either through the lymphatic vessels or blood vessels. Meanwhile, the fluorescent micelles were not detected in healthy lymph nodes (Supplementary Fig. S4). The histological examinations of the enhanced accumulation of DACHPt/m in the metastatic lymph nodes are quite consistent with the significant growth inhibition of lymph node metastasis observed in DACHPt/m treated animals (Fig. 3).

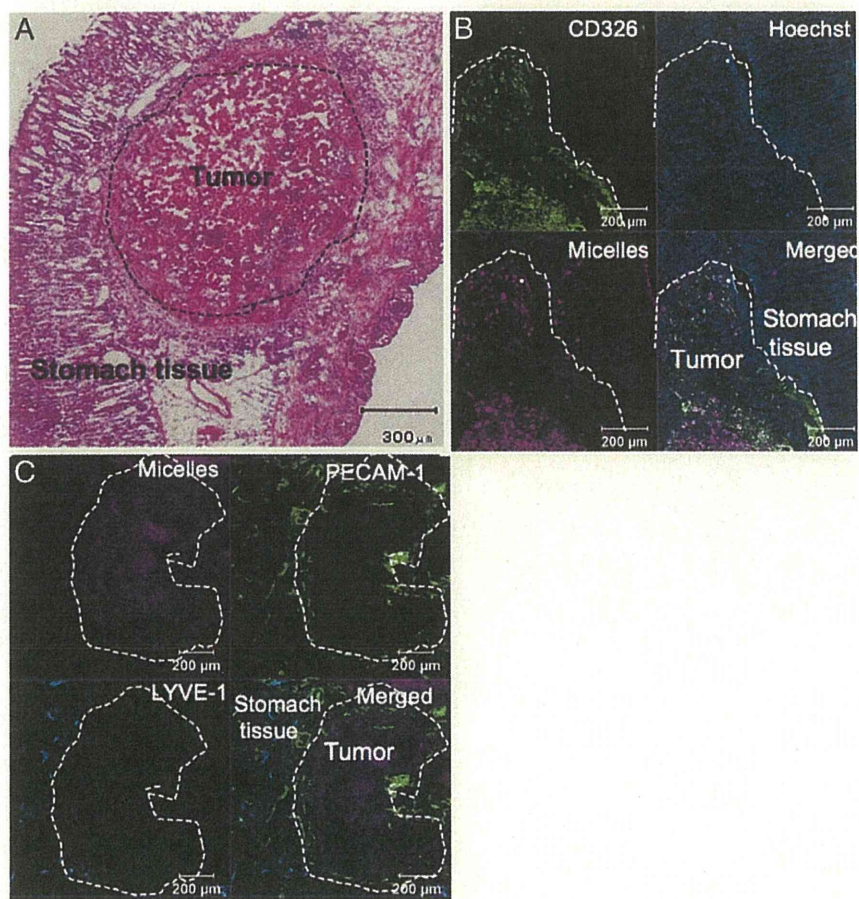
## 4. Discussion

In the present study, we examined the targeting ability of DACHPt/m against the orthotopic model of scirrhous gastric cancer (SGC) from OCUM-2MLN cells, which is accompanied with very high rate of lymph node metastasis [30–31]. The metastasis to the lymph nodes is an important indicator for the staging of SGC and a determinant for the prognosis [35–36]. Here, we have successfully demonstrated that systemically administered DACHPt/m can target both orthotopic scirrhous gastric tumors and their lymphatic metastasis, achieving a remarkable inhibitory effect on their growth.

The biodistribution study revealed that the micelles significantly accumulated in the tumors and the metastatic lymph nodes (Figs. 5 and 6) while showing appreciably lower accumulation in organs or healthy lymph nodes (Fig. 5 and Fig. S4). The accumulation of DACHPt/m in the orthotopic OCUM-2MLN tumors might be related to the passive targeting based on the EPR effect. However, we have previously reported that doxorubicin-loaded polymeric micelles with 65-nm diameter showed poor accumulation and reduced efficacy against orthotopic OCUM-2MLN tumors [9]. Regarding such discrepancy, we assume that a relatively small size (30 nm) of DACHPt/m should be important for effective extravasation and tumor penetration in orthotopic OCUM-2MLN tumors. Recently, we have studied the size effect of DACHPt/m in subcutaneous human pancreatic adenocarcinoma BxPC3 tumors [29], which share histological characteristics with OCUM-2MLN tumors, such as sparse formation of blood vessels and very thick fibrotic stroma [30–31]. Accordingly, DACHPt/m with 30-nm diameter effectively extravasated and penetrated in the pancreatic tumors while DACHPt/m with diameter larger than 50-nm remained in the perivascular areas of the tumors [29]. Based on these observations, we preliminarily studied the size effect of DACHPt/m in an orthotopic OCUM-2MLN tumor model in this study (Supplementary Information). As shown in Supplementary Fig. S3, 30-nm DACHPt/m showed potent antitumor efficacy and enhanced tumor accumulation against gastric tumors, whereas 70-nm DACHPt/m failed to exhibit significant antitumor effect and showed

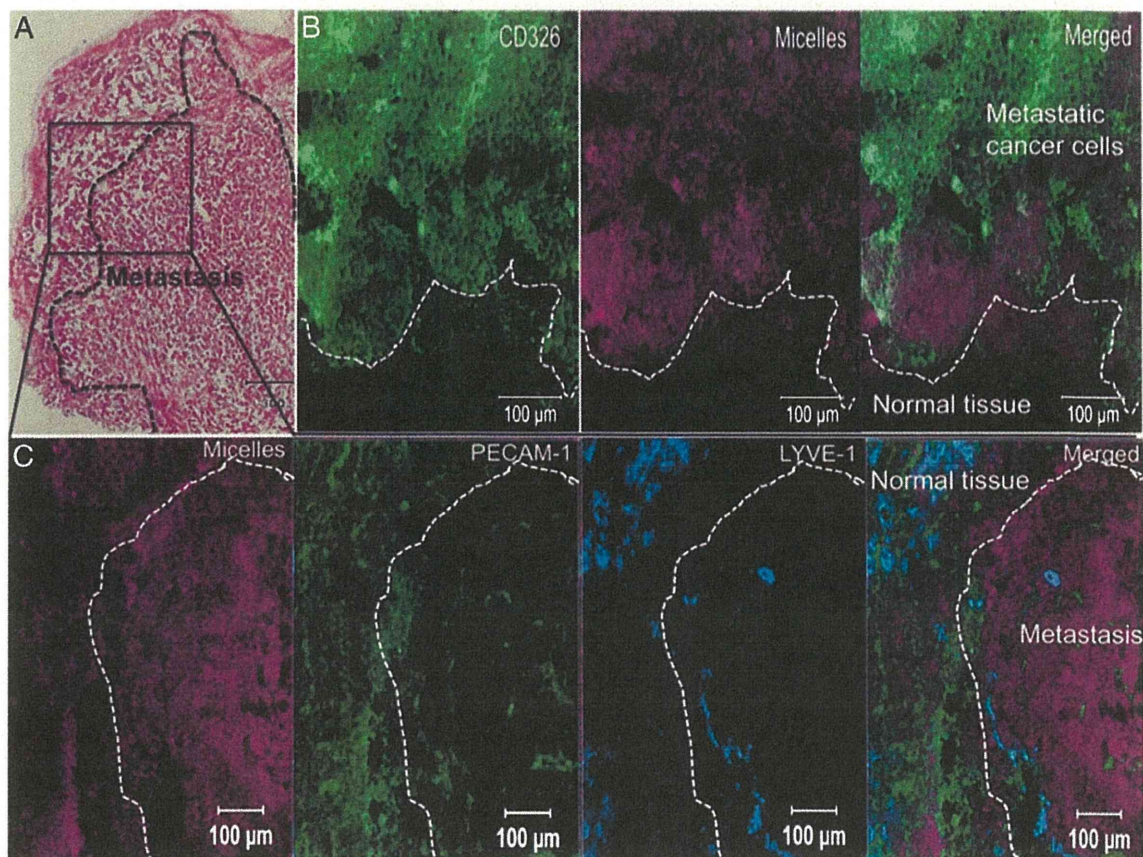


**Fig. 5.** Biodistribution of Alexa 680-labeled DACHPt/m. A, Whole body near infrared (NIR) fluorescent image of orthotopic OCUM-2-MLN-Luc tumor-bearing mouse 24 h after the injection of Alexa 680-labeled DACHPt/m. The micelles are detected specifically at the tumor site. B, Ex vivo fluorescent imaging of orthotopic tumor, organs and metastatic lymph nodes.



**Fig. 6.** Histological analysis of orthotopic OCUM-2-MLN-Luc and microdistribution of fluorescent labeled DACHPt/m. A, H&E staining of orthotopic OCUM-2-MLN-Luc tumor sections. B, Immunofluorescence microscopy of gastric cancer cells (CD326, green) and Alexa 594-labeled DACHPt/m (magenta) in orthotopic OCUM-2-MLN-Luc tumors. The cells nuclei were stained with Hoechst 33342 (blue). C, Microdistribution of Alexa 594-labeled DACHPt/m (micelles, magenta), blood vessels (PECAM-1, green) and lymphatic vessels (LYVE-1, blue) in orthotopic OCUM-2-MLN-Luc tumors determined by immunofluorescence microscopy.

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**Fig. 7.** Histological analysis of metastatic lymph nodes and microdistribution of fluorescent labeled DACHPt/m. A, H&E staining of metastatic lymph node. B, Immunofluorescence microscopy of gastric cancer cells (CD326, green) and Alexa 594-labeled DACHPt/m (micelles, magenta) in the metastatic lymph nodes. C, Microdistribution of Alexa 594-DACHPt/m (micelles, magenta), blood vessels (PECAM-1, green) and lymphatic vessels (LYVE-1, blue) in metastatic lymph nodes (Inset of A) determined by immunofluorescence microscopy.

poor tumor accumulation. These results are consistent with the aforementioned assumption that effective properties of DACHPt/m against a scirrhous gastric cancer model may be attributed to their relatively small size.

The observation of DACHPt/m accumulation into the fibrous OCUM-2MLN tumor here is apparently consistent with the scheme of EPR effect. On the other hand, the mechanisms of the accumulation of the micelles in the metastatic lymph nodes remain to be clarified yet. Two mechanisms for the accumulation of the micelles in the metastatic lymph nodes may be proposed: (i) the micelles accumulate in the orthotopic tumors, followed by migration and accumulation in the metastatic lymph nodes via the lymphatic vessels, (ii) the micelles can directly accumulate in the metastatic lymph nodes via blood vessels in the metastatic niche probably due to the enhanced permeability of these blood vessels. Regarding the former mechanism, intratumorally-injected nanocarriers have been demonstrated to accumulate in the metastatic lymph nodes [16,17,37,38]. Also, Harisinghani et al. reported in patients that systemically injected superparamagnetic iron oxide nanoparticles (SPION) accumulate in the metastatic lymph nodes [39]. This phenomenon was explained by accumulation of SPION in solid tumors, followed by their uptake by tumor macrophages, which migrate to metastatic lymph nodes [39]. The detailed mechanisms underlying the accumulation of the micelles in the metastatic lymph nodes are under investigation and will be reported elsewhere.

The targeting capability of the micelles to scirrhous gastric tumors and their metastatic lymph nodes could be applied not only for treatment but also for diagnosis. A variety of contrast agents, including

fluorescent probes, MRI or PET contrast agents [5] can be incorporated into the micellar structure. Thus, primary scirrhous tumors and nodal involvement may be directly observed by non-invasive imaging. Moreover, the combination of therapy and imaging within single micelles may allow the evaluation of the therapeutic response, offering an emerging concept of theranostic nanomedicines [5,27].

In conclusion, our results highlight that systemically injected DACHPt/m can extravasate and penetrate in orthotopic scirrhous gastric tumors and lymph node metastasis, eliciting significantly potent antitumor activity. Enhanced drug delivery to the lymph node metastasis by polymeric micelles can improve the morbidity of the patients with SGC. DACHPt/m can also be useful for the adjuvant therapy of SGC, that is, the administration of the micelles before surgery, by improving the lymph node status while controlling the tumor volume, which may lead to the downgrading of unresectable scirrhous gastric cancer. Moreover, control of distant lymph node metastasis by DACHPt/m can impede further dissemination of the disease. Our findings suggest the high potential of systemically administered nanocarriers for the treatment of scirrhous gastric cancer as well as lymph node metastasis.

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