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Viral protein-coating of magnetic nanoparticles using simian virus 40 VP1

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ABSTRACT

Artificial beads including magnetite and fluorescence particles are useful to visualize pathologic tissue, such as cancers, from harmless types by magnetic resonance imaging (MRI) or fluorescence imaging. Desirable properties of diagnostic materials include high dispersion in body fluids, and the ability to target specific tissues. Here we report on the development of novel magnetic nanoparticles (MNPs) intended for use as diagnosis and therapy that are coated with viral capsid protein VP1-pentamers of simian virus 40, which are monodispersive in body fluid by conjugating epidermal growth factor (EGF) to VP1. Critically, the coating of MNPs with VP1 facilitated stable dispersion of the MNPs in body fluids. In addition, EGF was conjugated to VP1 coating on MNPs (VP1-MNPs). EGF-conjugated VP1-MNPs were successfully used to target EGF receptor-expressing tumor cells *in vitro*. Thus, using viral capsid protein VP1 as a coating material would be useful for medical diagnosis and therapy.

1. Introduction

Simian virus 40 (SV40) is a small non-enveloped DNA virus of the *Polyomaviridae* family. The capsid of SV40 is 45 nm in diameter and composed mainly of 72 copies of pentamers of the major capsid protein, VP1 (Liddington et al., 1991). When expressed in insect cells using recombinant baculovirus, VP1 self-assembles into virus-like particles (VLPs) of 45 nm in diameter (Kosukegawa et al., 1996). VLPs isolated from the cells are disassembled *in vitro* into VP1-pentamers by the addition of DTT and EGTA (Ishizu et al., 2001). These VP1-pentamers self-reassemble *in vitro* into 45-nm VLPs under appropriate conditions (Kanesashi et al., 2003; Kawano et al., 2006). During the reassembly, VLPs can encapsulate materials such as DNA (Enomoto et al., 2011; Tsukamoto et al., 2007) and proteins (Inoue et al., 2008). Furthermore, it is possible to confer cell-type specificity to VLPs by inserting foreign peptides (Takahashi et al., 2008) or conjugating human epidermal growth factor (EGF) (Kitai et al., 2011) to VP1. Thus, VP1 of SV40 is potentially very useful as a medical nanomaterial.

Magnetic nanoparticles (MNPs) mainly composed of iron oxide or gadolinium have been developed as MRI contrast agents—materials that reduce the relaxation time of protons (Weinmann et al., 2003). We have previously prepared and reported on the properties of size-controlled and water-dispersed citrate-coated MNPs (CMNPs) (Hatakeyama et al., 2011). Due to their monodispersity in water, we expected that the CMNPs would be suitable as an MRI contrast agent (Hatakeyama et al., 2011). However, CMNPs form aggregates in a physiological salt condition (this study). In addition to dispersibility, the targeted delivery

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and retention of nanoparticles to specific tissues is an important requirement for the development of MRI contrast agents for diagnosis. Since early MRI contrast agents diffuse well into intravascular and interstitial spaces, there is increasing demand for an agent with a tissue-specific diffusion distribution to improve diagnostic accuracy (Weinmann et al., 2003). In particular, the development of an agent that accumulates highly and specifically in malignant tumors is of interest for clinicians because this may allow an accurate diagnosis in early stages of the disease. To this aim, recently various types of MNPs, such as labeled with tumor-specific monoclonal antibody (Anderson et al., 2000; Gohr-Rosenthal et al., 1993; Remsen et al., 1996; Shahbazi-Gahrouei et al., 2001; Sipkins et al., 1998; Suwa et al., 1998) or presented tumor-selective peptide (Montet et al., 2006; Nasongkla et al., 2006; Uchida et al., 2006), have been synthesized.

Here, we present the synthesis of novel CMNPs that were coated with VP1-pentamers of simian virus 40, which produced CMNPs that were highly dispersive, and that attached at targeted cells by conjugating EGF to VP1. VP1 coating allowed stable dispersion of CMNPs and prevented their aggregation. EGF conjugation to VP1 enabled the selective targeting of the particles to EGF receptor (EGFR) on the cells *in vitro*, which is overexpressed in a variety of tumors (Normanno et al., 2003; Salomon et al., 1995). Thus, highly dispersible CMNPs with cell-targeting ability would be applicable for the diagnosis of tumors by MRI.

2. Materials and methods

2.1. Preparation of VP1 pentamers

$VLPs^{WT}$ and $VLPs^{N138C}$ were prepared as described previously (Ishizu et al., 2001). Briefly, recombinant baculovirus expressing either $VLPs^{WT}$ or $VLPs^{N138C}$ was generated using the baculovirus expressing system (Invitrogen) and Sf-9 cells were infected with these viruses. $VLPs^{WT}$ and $VLPs^{N138C}$ were purified from lysates of virus-infected Sf-9 cells by cesium chloride density gradient and dialyzed in dialysis buffer containing 20 mM Tris–HCl (pH 7.9), 150 mM NaCl and 0.1% NP-40. Purified $VLPs^{WT}$ and $VLPs^{N138C}$ were dissociated in buffer containing 5 mM DTT and 5 mM EGTA, and the dissociated samples were fractionated by gel filtration (Superdex 200, GE Healthcare) to collect homogeneous $VP1^{WT}$- and $VP1^{N138C}$-pentamers. For preparation of $VP1^{\Delta C58}$-pentamers, the C-terminal 58 amino-acids were deleted from VP1 and the histidine tag sequence was fused to the C-terminus of $VP1^{\Delta C58}$. Sf-9 cells were then infected with recombinant baculovirus expressing $VP1^{\Delta C58}$. $VP1^{\Delta C58}$-pentamers were purified from cell lysates by nickel-nitrilotriacetic acid agarose resin (Qiagen) according to the manufacture's instruction. $VP1^{\Delta C58}$-pentamers were further purified by Superose 6 chromatography (GE Healthcare), and then gel fractionation.

2.2. Construction of VP1-CMNPs

CMNPs were prepared as described before (Hatakeyama et al., 2011). CMNPs were mixed with either $VP1^{WT}$-pentamers, $VP1^{N138C}$-pentamers or $VP1^{\Delta C58}$-pentamers at a molar ratio of 1:72. The mixture was dialyzed in coating buffer containing 20 mM MOPS-NaOH (pH 7.0), 150 mM NaCl, and 2 mM $CaCl_2$. To remove free pentamers, the sample was centrifuged at 20,400 × *g* for 15 min at 4 °C. The pellet fraction containing VP1-CMNPs was washed twice and resuspended in coating buffer. For TEM observation, the samples were negatively stained with 2% ammonium molybdate and were observed using a TEM (H-7500, Hitachi) as described previously (Ishizu et al., 2001). The molar of CMNPs was calculated from the Fe^{2+} concentrations in CMNPs suspension and the molecular weight of CMNPs which was estimated from the diameter of its.

2.3. Measurement of diameter and electric potential of nanoparticles

Diameters and zeta potentials of intact CMNPs and $VP1^{WT}$-CMNPs in various solutions were analyzed using Zeta-potential & Particle size Analyzer (ELSZ-2, Otsuka Electronics Co. Ltd., Osaka, Japan) according to the manufacture's protocol.

2.4. Relaxivities and T1- and T2-weighted MR images of VP1-CMNPs

The longitudinal and transverse relaxation times of the different sized CMNPs or VP1-CMNPs were measured in distilled water using a 0.47 T NMR Analyzer (NMS 120 Minispec, Bruker Optics, Ibaraki, Japan) at 40 °C. The longitudinal (r_1) and transverse (r_2) relaxivities of Fe were calculated from the measured relaxation times. The T1- and T2-weighted MR images of freucarbotran, gadopentetate dimeglumine, or 27 nm VP1-CMNPs were obtained using a 7-T horizontal MRI (Biospec, Avence-I, Bruker BioSpin, Germany) with a volume coil for transmission and reception (35 mm inner-diameter, Rapid Biomedical, Germany). Measurement parameters were as follows: spin echo sequence, TR/TE = 400.0/9.6 ms (T1-weighted imaging), or multi-echo spin echo sequence, TR/TE = 3000/60 ms (T2-weighted imaging), field-of-view = 38.4 × 38.4 mm^2, slice thickness = 2 mm, and matrix size = 256 × 256.

2.5. Sucrose gradient sedimentation analysis

Sucrose gradient sedimentation analyses were performed as described previously (Kawano et al., 2006). In brief, 20 μL of samples containing $VP1^{WT}$-CMNPs or $VP1^{\Delta C58}$-CMNPs were loaded onto the top of 600 μL of 20–40% sucrose gradient in 20 mM Tris–HCl (pH 7.9) in a 5 × 41-mm open-top tube and were centrifuged at 232,000 × *g* for 1 h at 4 °C in an SW55Ti rotor (Beckman). After centrifugation, 12 fractions were collected from the top of the gradient. Each fraction was separated by 10% SDS-PAGE, and was analyzed by Western blotting with anti-VP1 antibody (Ishizu et al., 2001).

2.6. Measurement of Fe^{2+} concentration in the blood

Mice were anesthetized with 2.0% isoflurane and were administrated *via* the tail vein with 200 μL of CMNPs or $VP1^{WT}$-CMNPs (7.5 mg/mL) in PBS. Fifty microliters of blood was collected from a femoral artery of mice at various time points after administration. Ten microliters of blood samples was mixed with 990 μL of HNO_3, heated in a microwave oven for 1 min, and then subjected to the ICP-OES analyzer, Prodigy ICP (Teledyne Leeman Labs Inc., NH) to measure the concentration of Fe^{2+} using yttrium as a standard.

2.7. EGF conjugation to VP1-CMNPs

Recombinant human EGF (Peprotech) was conjugated onto $VP1^{WT}$-CMNPs or $VP1^{N138C}$-CMNPs using the heterobifunctional crosslinker with N-hydroxysuccinimide ester and maleimide groups, $SM(PEG)_2$ (Thermo Scientific) as described before (Kitai et al., 2011). Briefly, EGF was incubated with the $SM(PEG)_2$ crosslinker in reaction buffer containing 20 mM MOPS-NaOH (pH 6.5) and 150 mM NaCl for 2 h at 4 °C. After incubation, EGF was quenched in quenching buffer containing 20 mM MOPS-NaOH (pH 6.5), 150 mM NaCl, and 100 mM ethanolamine for 2 h at 4 °C. To remove excess $SM(PEG)_2$, the reaction mixture was dialyzed in the