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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.

Keywords: Nanoparticle; Viral capsid protein; Diagnostic; Coating; Targeting

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 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^{ΔC58}-pentamers, the C-terminal 58 amino-acids were deleted from VP1 and the histidine tag sequence was fused to the

1 C-terminus of VP1^{ΔC58}. Sf-9 cells were then infected with recombinant baculovirus
2 expressing VP1^{ΔC58}. VP1^{ΔC58}-pentamers were purified from cell lysates by
3 nickel-nitrilotriacetic acid agarose resin (Qiagen) according to the manufacture's
4 instruction. VP1^{ΔC58}-pentamers were further purified by Superose 6 chromatography (GE
5 Healthcare), and then gel fractionation.

6 7 2.2. Construction of VP1-CMNPs

8 CMNPs were prepared as described before (Hatakeyama et al., 2011). CMNPs were
9 mixed with either VP1^{WT}-pentamers, VP1^{N138C}-pentamers or VP1^{ΔC58}-pentamers at a molar
10 ratio of 1:72. The mixture was dialyzed in coating buffer containing 20 mM MOPS-NaOH
11 (pH7.0), 150 mM NaCl, and 2 mM CaCl₂. To remove free pentamers, the sample was
12 centrifuged at 20,400 g for 15 min at 4°C. The pellet fraction containing VP1-CMNPs was
13 washed twice and resuspended in coating buffer. For TEM observation, the samples were
14 negatively stained with 2% ammonium molybdate and were observed using a TEM
15 (H-7500, Hitachi) as described previously (Ishizu et al., 2001). The molar of CMNPs was
16 calculated from the Fe²⁺ concentrations in CMNPs suspension and the molecular weight of
17 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 ferucarbotran, gadopentetate dimeglumine, or 27 nm VP1-CMNPs were obtained using a 7-tesla horizontal MRI (Biospec, Avenice-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 x 38.4 mm², slice thickness = 2 mm, and matrix size = 256 x 256.

2.5. Sucrose Gradient Sedimentation Analysis

Sucrose gradient sedimentation analyses were performed as described previously (Kawano et al., 2006). In brief, 20 μ L of samples containing VP1^{WT}-CMNPs or VP1 ^{Δ C58}-CMNPs were loaded onto the top of 600 μ L of 20% to 40% sucrose gradient in 20 mM Tris-HCl (pH 7.9) in a 5 $\times$ 41-mm open-top tube and were centrifuged at 232,000 $\times$ 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 Fe²⁺ Concentration in the Blood

Mice were anesthetized with 2.0% isoflurane and were administrated via the tail vein with 200 μ L of CMNPs or VP1^{WT}-CMNPs (7.5 mg/mL) in PBS. Fifty μ L of blood was collected from a femoral artery of mice at various time points after administration. Ten μ L of blood samples was mixed with 990 μ L of HNO₃, 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 Fe²⁺ 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 (pH6.5) and 150 mM NaCl for 2 h at 4°C. After incubation, EGF was quenched in quenching buffer containing 20 mM MOPS-NaOH (pH6.5), 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 reaction buffer for 16 h at 4°C. Then, the linker-conjugated EGF and VP1-CMNPs were mixed at 1.15 to 1 weight ratio and were incubated for 12 h at 4°C. After incubation, to quench the reaction, NaOH was added to the sample at a final concentration of 15 mM. The sample was mounted onto the top of 30 % sucrose containing PBS and centrifuged at 230,000 g for 15 min at 4°C in a Beckman SW55Ti rotor. The pellet fraction containing EGF-conjugated VP1^{WT}-CMNPs or VP1^{N138C}-CMNPs was resuspended in PBS.

2.8. Cell Binding Assay

The human epidermoid carcinoma cell line, A431 (Hirabayashi et al., 1999) and the human colon adenocarcinoma cell line, WiDr (Noguchi et al., 1979) were kindly provided

by Dr. M. Ueda (Keio University, Tokyo, Japan). A431 or WiDr cells (4×10^5 cells) were incubated with EGF-conjugated VP1^{WT}-CMNPs or VP1^{N138C}-CMNPs at an amount equivalent to 2 μ g of VP1 in the presence or absence of various amounts of free EGF for 1 h at 4°C in serum-free media. Free EGF was used to compete with EGF-conjugated nanoparticles for binding to the EGFR on the cell surface. Cells were collected, resuspended in SDS loading buffer, and then sonicated. Samples were separated by 10% SDS-PAGE, and Western analysis was performed with anti-VP1 (Ishizu et al., 2001) and anti-actin antibodies. Alternatively, sonicated samples were incubated with 100 μ L of 6N HCl for 30 min at 55°C, and were then subjected to ICP-OES analyzer, Prodigy ICP (Teledyne Leeman Labs Inc, NH) to measure the Fe²⁺ concentration using yttrium as a standard.

3. Results and discussion

3.1. Synthesis of CMNPs Coated with VP1.

We have previously reported on the synthesis of water-dispersible CMNPs for use as an MRI contrast agent (Hatakeyama et al., 2011). For use *in vivo*, CMNPs should exhibit stable dispersibility in body fluid. To give the CMNPs an ability of active targeting to cells, here we aimed to synthesize CMNPs encapsulated with SV40-VLPs of 45 nm in diameter.

1 As SV40-VLPs are dispersible *in vivo* and their surfaces can be easily modified by genetic
2 or chemical methods, it led us to expect that encapsulation of CMNPs with SV40-VLPs
3 would also be suitable for active targeting. To evaluate whether the CMNPs were
4 encapsulated in SV40-VLPs, we used CMNPs with diameters of 8, 20, and 27 nm. Firstly,
5 the CMNPs were mixed with wild-type VP1-pentamers (VP1^{WT}-pentamers) (the basic
6 subunit of SV40-VLP) in a reaction buffer solution, and the mixtures were then dialyzed
7 against a physiological buffer. Transmission electron microscope (TEM) measurements
8 showed that all of the different-sized CMNPs mixed with VP1^{WT}-pentamers were covered
9 with lightly stained layers of 5 nm in thickness (Fig. 1b, lower panels), compared to
10 untreated CMNPs (Fig. 1b, upper panels). These results showed that VP1^{WT} -pentamer did
11 not assemble into the 45-nm SV40-VLPs but coated CMNPs with a monolayer of VP1^{WT}
12 -pentamer in a CMNP's size-dependent manner. Thus, coating of CMNPs with the VP1^{WT}
13 -pentamer occurred instead of encapsulating CMNPs in VLPs. CMNPs coated with VP1^{WT}
14 (VP1^{WT}-CMNPs) were monodispersed under physiological salt conditions (Fig. 1b, lower
15 panels), whereas control CMNPs formed aggregates in this buffer (Fig. 1b, upper panels).
16 To further examine the dispersibility of CMNPs, we measured the size distribution of the
17 particles in solution using dynamic light scattering (DLS). The average sizes of
18 VP1^{WT}-CMNPs were approximately 10 nm larger than the diameters of original CMNPs

(Fig. 1c), confirming that CMNPs of any given size could be coated with VP1^{WT}-pentamers. In contrast, the sizes of the control CMNPs in solution were roughly 500 nm, showing uncoated CMNPs to aggregation. Taken together, these findings demonstrated that VP1 coating yielded monodisperse CMNPs under physiological salt conditions.

To directly examine whether the VP1 coating affected the surface properties of CMNPs, we measured the electric potential of their surfaces (Fig. 1d). Due to the citrate-coating, the surfaces of CMNPs were strongly charged with -18 mV at pH 7. In contrast, the surfaces of VP1^{WT}-CMNPs had a weak negative charge of -4 mV, indicating that VP1 coating changed the surface potential of CMNPs. Notably, this finding was supported by experiments showing that the surface potential of VLPs was comparable to that of VP1^{WT}-CMNPs.

To characterize the properties of VP1-CMNP as an MRI contrast agent, we measured the relaxivities (r_1 , r_2) for different sized (8, 20 and 27 nm) CMNPs with and without VP1-coating in distilled water. The relaxivities of these VP1-CMNPs or CMNPs are shown in Table 1. Generally, at 0.47-tesla VP1-coating decreased the r_1 of the CMNPs without drastically affecting r_2 . The subsequent increase in the r_2/r_1 ratio enhanced the potential of VP1-coated CMNPs as a T2 contrast agent. The r_1 for the 27 nm VP1-CMNPs was similar to that for conventional MRI contrast agents (ferucarbotran, ResovistTM, $r_1 = 24.7 \text{ s}^{-1} \text{ mM}^{-1}$)

1 and the r_2 was large compared to that of ferucarbotran ($r_2 = 160.0 \text{ s}^{-1} \text{ mM}^{-1}$). We also
2 acquired T1- and T2-weighted MR images of 27 nm VP1-CMNPs at 7-tesla MRI with
3 Gd-DTPA (gadopentetate dimeglumine, Magnevist™) and ferucarbotran (Fig. 1e). The
4 signal intensity for VP1-CMNP in both the T1- and T2-weighted images gradually
5 decreased with the VP1-CMNP concentration. The signal reduction for the VP1-CMNPs
6 was larger than that for ferucarbotran on both T1- and T2-weighted images. From these
7 results it is possible to speculate that VP1-CMNPs have better capability as a T2 contrast
8 agent than conventional ferucarbotran.

9 The C-terminal region of VP1 is important for pentamer-pentamer interaction between
10 VP1-pentamers during the assembly of SV40-VLPs (Garcea et al., 1987; Yokoyama et al.,
11 2007). Using a VP1 mutant with a 58 amino-acid deletion in the C-terminal region
12 (VP1^{ΔC58}), we investigated whether the pentamer-pentamer interaction would be required
13 for the VP1 coating on the surface of CMNPs. CMNPs were mixed with either
14 VP1^{WT}-pentamers or VP1^{ΔC58}-pentamers, and were then examined under the TEM. As
15 shown in Fig. 2a, lightly stained layers 5 nm-thick were around CMNPs regardless of
16 whether the VP1^{WT} or the VP1^{ΔC58} was used, suggesting that the pentamer-pentamer
17 interaction was not necessary for the attachment of VP1-pentamers onto the surfaces of
18 CMNPs. Unlike VP1^{WT}-CMNPs (Fig. 2a, left panel), however, VP1^{ΔC58}-attached CMNPs

1 formed particulate aggregates in physiological buffer solution (Fig. 2a, right panel). We
2 have previously reported that DNA is coated with VP1^{WT}-pentamers and therefore
3 promotes the assembly of VP1^{WT}-pentamers into 45 nm spherical particles (Tsukamoto et
4 al., 2007). Time-course analysis of this DNA-mediated assembly suggests that
5 VP1^{WT}-pentamers form aggregates within the first 1 h of reaction, and then form spherical
6 particles through the interaction between VP1^{WT}-pentamers in the next 1 h (Tsukamoto et
7 al., 2007). From this point of view, VP1^{ΔC58}-attached CMNPs lacking the
8 pentamer-pentamer interaction may correspond to VP1^{WT}-attached DNA in the early stages
9 of the reaction. To examine the dispersibility of VP1^{ΔC58}-attached CMNPs under
10 physiological conditions, we carried out sucrose gradient sedimentation analysis (Fig. 2b)
11 (Kawano et al., 2006). After centrifugation, 12 fractions were collected from the top of the
12 gradient. VP1^{WT}-CMNPs were found in fractions 5–9 (Fig. 2b, upper panel), whereas
13 VP1^{ΔC58}-attached CMNPs were detected in the bottom fraction (Fig. 2b, lower panel),
14 suggesting the aggregation of the nanoparticles. These results imply that the
15 pentamer-pentamer interaction is required for the formation of dispersible VP1^{WT}-CMNPs.

17 3.2. Dispersibility of VP1-CMNPs in Serum.

18 Practical applications of **diagnosis** necessitate their dispersibility in body fluids.

1 Therefore, we examined the degree to which VP1^{WT}-CMNPs would disperse in serum.
2 VP1^{WT}-CMNPs and control CMNPs were incubated in either phosphate buffered saline
3 (PBS) or fetal bovine serum (FBS). In agreement with the data in Fig. 1b and c,
4 VP1^{WT}-CMNPs were highly dispersible in PBS, while the control CMNPs aggregated
5 together (Fig. 3a). Surprisingly, VP1^{WT}-CMNPs were still dispersible even in FBS (Fig. 3a)
6 although intact CMNPs formed aggregates (Fig. 3a). To verify the TEM results, we
7 measured the diameter of CMNPs. DLS analysis showed that the diameter of
8 VP1^{WT}-CMNPs was approximately 40 nm in both PBS and FBS (Fig. 3b), indicating that
9 VP1^{WT}-CMNPs were monodispersed in FBS as well as PBS. On the other hand, the control
10 CMNPs in PBS and FBS were 400 and 150 nm in diameter, respectively (Fig. 3b), showing
11 that the water-dispersible, control CMNPs did not disperse in either PBS or FBS. To verify
12 the dispersibility of VP1^{WT}-CMNPs *in vivo*, mice were administrated intravenously with an
13 equal dose of CMNPs or VP1^{WT}-CMNPs, and the concentration of CMNPs-derived Fe²⁺ in
14 the blood was measured at various times. We expected that highly dispersible
15 VP1^{WT}-CMNPs would keep drifting in the blood stream, whereas aggregated CMNPs
16 would tend to settle down and to be quickly uptaken in the liver. As shown in Fig. 3c, the
17 basal Fe²⁺ concentration in both cases was approximately 0.5 mg/μL just before injection.
18 Moreover, we observed rapid increases in Fe²⁺ concentration in both cases just after

1 injection. However, the Fe^{2+} concentration in VP1^{WT}-CMNPs-injected mice was
2 approximately twice of that in CMNPs-injected mice at this point in time (Fig. 3c).
3 Furthermore, the Fe^{2+} concentration in both cases gradually decreased to basal level. In
4 addition, the Fe^{2+} concentration decreased much slower in VP1^{WT}-CMNPs-injected mice (a
5 half-life of 45 min) than in CMNPs-injected mice (a half-life of 45 sec; Fig. 3c), indicating
6 the longer retention time of VP1^{WT}-CMNPs in blood. These results strongly suggest that
7 VP1^{WT}-CMNPs are dispersive *in vivo*. In this study, the profound liver uptake of
8 aggregated CMNPs might induce quick decline of CMNPs-derived Fe^{2+} concentration in
9 the blood.

11 3.3. Targeting of EGFR-Overexpressing Tumor Cells by EGF-Conjugated VP1-CMNPs.

12 Because EGFR is overexpressed on the surface of various tumor cells (Normanno et
13 al., 2003; Salomon et al., 1995), it is possible to target certain tumor cells through EGFR.
14 We previously constructed SV40-VLPs conjugated with EGF using the heterobifunctional
15 crosslinker, SM(PEG)₂ and VP1^{N138C}, in which an asparagine (N) residue located on the
16 outside surface of VLPs (Takahashi et al., 2008) was substituted to a cysteine (C) residue
17 (Kitai et al., 2011). The EGF-conjugated SV40-VLPs were shown to selectively target
18 EGFR-expressing tumor cells *in vitro* (Kitai et al., 2011). Since SM(PEG)₂ preferably

1 crosslinks between a primary amine of EGF and the cysteine residue of VLPs^{N138C}, EGF is
2 considered to be selectively attached to the outside surface of VLPs^{N138C}. Moreover,
3 although VP1^{WT} has a partially accessible cysteine residue on the surface of VP1-pentamer,
4 VP1^{N138C} has additional cysteine residue on the surface loop of VP1. It leads us to expect
5 that EGF might be more functionally present on the surface of VP1^{N138C} than VP1^{WT}. In
6 addition, VLPs^{WT} generally bind to a variety of cells through the interaction between the
7 BC, DE, and HI loops of VP1 and GM1 ganglioside on the cells (Takahashi et al., 2008).
8 However, the mutation at position 138 elicits the conformational change of DE loop, and
9 thereby disrupts the binding of EGF-conjugated VLPs^{N138C} to various cells without EGFR
10 expression. Consequently, EGF-conjugated VLPs^{N138C} may be engaged exclusively in
11 EGF-specific targeting.

12 Based on these data (Kitai et al., 2011) and ideas, VP1^{N138C}-coated 27-nm CMNPs
13 (VP1^{N138C}-CMNPs) were synthesized, and EGF conjugated to both VP1^{WT}-CMNPs and
14 VP1^{N138C}-CMNPs using the crosslinker (Fig. 4a). By calculating the intensity of the protein
15 bands from Fig. 4b, the percentage of EGF conjugated to VP1^{N138C} protein in total VP1^{N138C}
16 protein of VP1^{N138C}-CMNPs appeared to be 26% only slightly larger than that of 18% in
17 VP1^{WT}-CMNPs, suggesting that VP1^{N138C} presents more functionally EGF than VP1^{WT}.
18 VP1^{N138C}-CMNPs were found to be monodispersed in physiological buffer regardless of

1 EGF conjugation (Fig. 4c).

2 To assess the specificity and efficiency of tumor-targeting by EGF-conjugated
3 VP1^{N138C}-CMNPs, we performed cell binding assay using two human tumor cell lines,
4 A431 (Hirabayashi et al., 1999) and WiDr (Noguchi et al., 1979), which express high and
5 low levels of EGFR, respectively. VP1^{WT}-CMNPs and VP1^{N138C}-CMNPs conjugated with
6 or without EGF were incubated with either A431 or WiDr cells in the presence or absence
7 of various concentrations of free EGF. Cells were then subjected to immunoblotting using
8 anti-VP1 antibody. As shown in Fig. 4d, both EGF-conjugated VP1^{WT}- and
9 VP1^{N138C}-CMNPs much more associated with A431 cells than those of EGF-unconjugated,
10 whereas the associations of WiDr cells with VP1^{WT}- and VP1^{N138C}-CMNPs were
11 comparable regardless of EGF conjugation. The association of A431 cells with
12 EGF-conjugated VP1^{N138C}-CMNPs was significantly inhibited by the addition of 0.3 µg
13 free EGF and competition was saturated (Fig. 4d, lanes 15-18). A similar inhibition pattern
14 was observed in the association between A431 cells and EGF-conjugated VP1^{WT}-CMNPs
15 (Fig. 4d, lanes 6-9). On the other hand, the association of WiDr cells with either
16 EGF-conjugated VP1^{WT}-CMNPs or VP1^{N138C}-CMNPs was hardly detected in this assay
17 (Fig. 4d, lanes 24-27 and 33-36). These results indicate that EGF-conjugated
18 VP1^{N138C}-CMNPs and VP1^{WT}-CMNPs specifically attach to tumor cells through the

EGF-EGFR interaction. It is noteworthy that the amounts of VP1^{N138C}-CMNPs attached to A431 cells (lane 15) were significantly larger than those of VP1^{WT}-CMNPs (lanes 6), suggesting that the VP1^{N138C}-CMNPs target EGFR-expressing tumor cells more effectively than the VP1^{WT}-CMNPs.

To further evaluate the selective association, we analyzed the amount of CMNPs-derived Fe²⁺ with A431 cells using the inductively coupled plasma optical emission spectrometry (ICP-OES) analyzer (Prodigy ICP). As shown in Fig. 4e, the conjugation of EGF to VP1^{WT}-CMNPs and VP1^{N138C}-CMNPs dramatically increased the amounts of Fe²⁺ associated with A431 cells. Consistent with the data in Fig. 4d, quantification of Fe²⁺ indicated that the amount of EGF-conjugated VP1^{N138C}-CMNPs bound to A431 cells was 3-fold larger than that of EGF-conjugated VP1^{WT}-CMNPs (Fig. 4e). Moreover, the addition of 0.3 µg free EGF to this system lowered the cell-associated levels of Fe²⁺ for both VP1^{WT}-CMNPs and VP1^{N138C}-CMNPs and the competition was saturated at this concentration. Remained Fe²⁺ amounts associated with A431 cells in the range of 1 and 3 µg free EGF concentration might be due to the EGFR-nonspecific binding of VP1^{WT}-CMNPs and VP1^{N138C}-CMNPs. Taken together, these data demonstrate that VP1^{N138C}-CMNPs are able to present EGF more effectively than VP1^{WT}-CMNPs so that the particles are targeted to EGFR-expressing tumor cells with remarkable specificity *in vitro*.

4. Conclusions

We showed that VP1 coating allows CMNPs to be monodispersed in body fluid such as serum and blood (Fig. 3a-c). This characteristic is very important for MRI contrast agents because monodispersible nanoparticles can reach tissues or organs by traveling in the blood stream. Since it has been suggested that viral capsid protein is a suitable material to achieve biocompatibility (Cormode et al., 2010), the VP1 coating may be also expected to provide biocompatibility to the nanoparticle and prevent their detection and elimination by the clearance mechanisms in the body. In addition, VP1 based architecture is shown to be very stable in broad range of salt concentration, pH, and temperature (Kawano et al., 2009) and considered to be safe for humans to use them as a vaccine material because there is no evidence that SV40 VP1 protein has acute or chronic cytotoxicity against human cells (Hilleman et al., 1998; Butel et al., 1999). Moreover, the VP1 coating allows further modification on the surface of CMNPs. As previously described (Bulte et al., 1994; Uchida et al., 2006), our results support that protein cages such as viral capsids or ferritins are promising platform for diagnosis use.

The most desirable feature of agents for diagnosis is the ability to target intended tissues or tumors *in vivo*. The distribution of nanoparticles in the body is affected by

multiple factors such as molecular size, shape and charge (Longmire et al., 2008). For instance, certain sizes of nanoparticles tend to be accumulated in tumor tissues through enhanced permeability and retention (Maeda et al., 2000), which is known as the enhanced permeability and retention (EPR) effect (Maeda, 2001). Apart from such passive targeting, active delivery and retention of nanoparticles into tissues or tumors is still far from reality although much effort has been devoted to designing probes that detect tumors. However, active targeting is of great interest to many clinicians because this technique will allow accurate diagnosis of tumor at an early stage. Tumor-specific monoclonal antibodies may be the ultimate tool for the active targeting. A number of investigators have studied this issue using small animals (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). However, considering the large amount of antibody used, this approach may be too costly for commercial use (Weinmann et al., 2003). On the other hand, it has been suggested that EGFR represents an ideal target for diagnosis and therapy of human carcinomas (Normanno et al., 2003; Salomon et al., 1995; Suwa et al., 1998). A majority of human solid tumors overexpress high levels of EGFR, and the overexpression level is often correlated with a poor prognosis (Salomon et al., 1995).

Because tumor cells are more heat-sensitive than normal cells, hyperthermia has

served as an anti-tumor therapy (Falk and Issels, 2001; Koga et al., 1983; van der Zee, 2002). Since MNPs can be used for hyperthermia as a heater element (Gupta and Gupta, 2005), EGF-conjugated VP1^{N138C}-CMNPs may be directly applicable to tumor-targeted hyperthermia therapy. That is, EGF-immobilized VP1^{N138C}-CMNPs has a great potential for an excellent therapeutic material as well as a diagnostic material.

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1 **References**

- 2 Anderson, S.A., Rader, R.K., Westlin, W.F., Null, C., Jackson, D., Lanza, G.M., Wickline,
3 S.A., Kotyk, J.J., 2000. Magnetic resonance contrast enhancement of neovasculature with
4 $\alpha(v)\beta(3)$ -targeted nanoparticles. *Magn. Reson. Med.* 44, 433-439.
- 5 Bulte, J.W., Douglas, T., Mann, S., Frankel, R.B., Moskowitz, B.M., Brooks, R.A.,
6 Baumgarner, C.D., Vymazal, J., Strub, M.P., Frank, J.A., 1994. Magnetoferritin:
7 characterization of a novel superparamagnetic MR contrast agent. *J. Magn. Reson. Imaging*
8 4, 497-505.
- 9 Butel, J. S., Lednicky J. A., 1999. Cell and molecular biology of simian virus 40: implications
10 for human infections and disease. *J. Natl. Cancer Inst.* 91, 119-134.
- 11 Enomoto, T., Kukimoto, I., Kawano, M.A., Yamaguchi, Y., Berk, A.J., Handa, H., 2011. *In*
12 *vitro* reconstitution of SV40 particles that are composed of VP1/2/3 capsid proteins and
13 nucleosomal DNA and direct efficient gene transfer. *Virology* 420, 1-9.
- 14 Falk, M.H., Issels, R.D., 2001. Hyperthermia in oncology. *Int. J. Hyperthermia* 17, 1-18.
- 15 Garcea, R.L., Salunke, D.M., Caspar, D.L., 1987. Site-directed mutation affecting
16 polyomavirus capsid self-assembly *in vitro*. *Nature* 329, 86-87.
- 17 Gohr-Rosenthal, S., Schmitt-Willich, H., Ebert, W., Conrad, J., 1993. The demonstration of
18 human tumors on nude mice using gadolinium-labelled monoclonal antibodies for magnetic

1 resonance imaging. Invest. Radiol. 28, 789-795.

2 Gupta, A.K., Gupta, M., 2005. Synthesis and surface engineering of iron oxide nanoparticles
3 for biomedical applications. Biomaterials 26, 3995-4021.

4 Hasegawa, S., Koshikawa-Yano, M., Saito, S., Morokoshi, Y., Furukawa, T., Aoki, I., Saga, T.,
5 2011. Molecular imaging of mesothelioma by detection of manganese-superoxide
6 dismutase activity using manganese-enhanced magnetic resonance imaging. Int. J. Cancer
7 128, 2138-2146.

8 Hatakeyama, M., Kishi, H., Kita, Y., Imai, K., Nishio, K., Karasawa, S., Masaike, Y.,
9 Sakamoto, S., Sandhu, A., Tanimoto, A., Gomi, T., Kohda, E., Abe, M., Handa, H., 2011. A
10 two-step ligand exchange reaction generates highly water-dispersed magnetic nanoparticles
11 for biomedical applications. J. Mater. Chem. 21, 5959-5966.

12 Hilleman, M. R., 1998. Discovery of simian virus 40 (SV40) and its relationship to
13 poliomyelitis virus vaccines. Dev. Biol. Stand. 94, 183-190.

14 Hirabayashi, K., Yano, J., Inoue, T., Yamaguchi, T., Tanigawara, K., Smyth, G.E., Ishiyama,
15 K., Ohgi, T., Kimura, K., Irimura, T., 1999. Inhibition of cancer cell growth by
16 polyinosinic-polycytidylic acid/cationic liposome complex: a new biological activity.
17 Cancer Res. 59, 4325-4333.

18 Inoue, T., Kawano, M.A., Takahashi, R.U., Tsukamoto, H., Enomoto, T., Imai, T., Kataoka, K.,

1 Handa, H., 2008. Engineering of SV40-based nano-capsules for delivery of heterologous
 2 proteins as fusions with the minor capsid proteins VP2/3. J. Biotechnol. 134, 181-192.
 3 Ishizu, K.I., Watanabe, H., Han, S.I., Kanesashi, S.N., Hoque, M., Yajima, H., Kataoka, K.,
 4 Handa, H., 2001. Roles of disulfide linkage and calcium ion-mediated interactions in
 5 assembly and disassembly of virus-like particles composed of simian virus 40 VP1 capsid
 6 protein. J. Virol. 75, 61-72.
 7 Kanesashi, S.N., Ishizu, K., Kawano, M.A., Han, S.I., Tomita, S., Watanabe, H., Kataoka, K.,
 8 Handa, H., 2003. Simian virus 40 VP1 capsid protein forms polymorphic assemblies *in*
 9 *vitro*. J. Gen. Virol. 84, 1899-1905.
 10 Kawano, M.A., Inoue, T., Tsukamoto, H., Takaya, T., Enomoto, T., Takahashi, R.U.,
 11 Yokoyama, N., Yamamoto, N., Nakanishi, A., Imai, T., Wada, T., Kataoka, K., Handa, H.,
 12 2006. The VP2/VP3 minor capsid protein of simian virus 40 promotes the *in vitro* assembly
 13 of the major capsid protein VP1 into particles. J. Biol. Chem. 281, 10164-10173.
 14 Kawano, M.A., Xing L., Tsukamoto, H., Inoue, T., Handa, H., Cheng, R.H., 2009. Calcium
 15 bridge triggers capsid disassembly in the cell entry process of simian virus 40. J. Biol.
 16 Chem. 284, 34703-34712.
 17 Kitai, Y., Fukuda, H., Enomoto, T., Asakawa, Y., Suzuki, T., Inouye, S., Handa, H., 2011. Cell
 18 selective targeting of a simian virus 40 virus-like particle conjugated to epidermal growth

1 factor. *J. Biotechnol.* 155, 251-256.

2 Koga, S., Izumi, A., Maeta, M., Shimizu, N., Osaki, Y., Kanayama, H., 1983. The effects of
3 total-body hyperthermia combined with anticancer drugs on immunity in advanced cancer
4 patients. *Cancer* 52, 1173-1177.

5 Kosukegawa, A., Arisaka, F., Takayama, M., Yajima, H., Kaidow, A., Handa, H., 1996.
6 Purification and characterization of virus-like particles and pentamers produced by the
7 expression of SV40 capsid proteins in insect cells. *Biochim. Biophys. Acta.* 1290, 37-45.

8 Liddington, R.C., Yan, Y., Moulai, J., Sahli, R., Benjamin, T.L., Harrison, S.C., 1991.
9 Structure of simian virus 40 at 3.8-Å resolution. *Nature* 354, 278-284.

10 Longmire, M., Choyke, P.L., Kobayashi, H., 2008. Clearance properties of nano-sized
11 particles and molecules as imaging agents: considerations and caveats. *Nanomedicine*
12 (Lond) 3, 703-717.

13 Maeda, H., 2001. The enhanced permeability and retention (EPR) effect in tumor vasculature:
14 the key role of tumor-selective macromolecular drug targeting. *Adv. Enzyme Regul.* 41,
15 189-207.

16 Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and
17 the EPR effect in macromolecular therapeutics: a review. *J. Control. Release* 65, 271-284.

18 Montet, X., Montet-Abou, K., Reynolds, F., Weissleder, R., Josephson, L., 2006. Nanoparticle

1 imaging of integrins on tumor cells. *Neoplasia* 8, 214-222.

2 Nasongkla, N., Bey, E., Ren, J., Ai, H., Khemtong, C., Guthi, J.S., Chin, S.F., Sherry, A.D.,
3 Boothman, D.A., Gao, J., 2006. Multifunctional polymeric micelles as cancer-targeted,
4 MRI-ultrasensitive drug delivery systems. *Nano Lett.* 6, 2427-2430.

5 Noguchi, P., Wallace, R., Johnson, J., Earley, E.M., O'Brien, S., Ferrone, S., Pellegrino, M.A.,
6 Milstien, J., Needy, C., Browne, W., Petricciani, J., 1979. Characterization of the WIDR: a
7 human colon carcinoma cell line. *In Vitro* 15, 401-408.

8 Normanno, N., Maiello, M.R., De Luca, A., 2003. Epidermal growth factor receptor tyrosine
9 kinase inhibitors (EGFR-TKIs): simple drugs with a complex mechanism of action? *J. Cell*
10 *Physiol.* 194, 13-19.

11 Remsen, L.G., McCormick, C.I., Roman-Goldstein, S., Nilaver, G., Weissleder, R., Bogdanov,
12 A., Hellstrom, I., Kroll, R.A., Neuwelt, E.A., 1996. MR of carcinoma-specific monoclonal
13 antibody conjugated to monocrystalline iron oxide nanoparticles: the potential for
14 noninvasive diagnosis. *AJNR Am. J. Neuroradiol.* 17, 411-418.

15 Salomon, D.S., Brandt, R., Ciardiello, F., Normanno, N., 1995. Epidermal growth
16 factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol.*
17 *Hematol.* 19, 183-232.

18 Shahbazi-Gahrouei, D., Williams, M., Rizvi, S., Allen, B.J., 2001. *In vivo* studies of

1 Gd-DTPA-monoclonal antibody and gd-porphyrins: potential magnetic resonance imaging
2 contrast agents for melanoma. J. Magn. Reson. Imaging 14, 169-174.

3 Sipkins, D.A., Cheresch, D.A., Kazemi, M.R., Nevin, L.M., Bednarski, M.D., Li, K.C., 1998.
4 Detection of tumor angiogenesis *in vivo* by alphaVbeta3-targeted magnetic resonance
5 imaging. Nat. Med. 4, 623-626.

6 Suwa, T., Ozawa, S., Ueda, M., Ando, N., Kitajima, M., 1998. Magnetic resonance imaging of
7 esophageal squamous cell carcinoma using magnetite particles coated with anti-epidermal
8 growth factor receptor antibody. Int. J. Cancer 75, 626-634.

9 Takahashi, R.U., Kaneshashi, S.N., Inoue, T., Enomoto, T., Kawano, M.A., Tsukamoto, H.,
10 Takeshita, F., Imai, T., Ochiya, T., Kataoka, K., Yamaguchi, Y., Handa, H., 2008.
11 Presentation of functional foreign peptides on the surface of SV40 virus-like particles. J.
12 Biotechnol. 135, 385-392.

13 Tsukamoto, H., Kawano, M.A., Inoue, T., Enomoto, T., Takahashi, R.U., Yokoyama, N.,
14 Yamamoto, N., Imai, T., Kataoka, K., Yamaguchi, Y., Handa, H., 2007. Evidence that SV40
15 VP1-DNA interactions contribute to the assembly of 40-nm spherical viral particles. Genes
16 Cells 12, 1267-1279.

17 Uchida, M., Flenniken, M.L., Allen, M., Willits, D.A., Crowley, B.E., Brumfield, S., Willis,
18 A.F., Jackiw, L., Jutila, M., Young, M.J., Douglas, T., 2006. Targeting of cancer cells with

- 1 ferrimagnetic ferritin cage nanoparticles. *J. Am. Chem. Soc.* 128, 16626-16633.
- 2 van der Zee, J., 2002. Heating the patient: a promising approach? *Ann. Oncol.* 13, 1173-1184.
- 3 Weinmann, H.J., Ebert, W., Misselwitz, B., Schmitt-Willich, H., 2003. Tissue-specific MR
- 4 contrast agents. *Eur. J. Radiol.* 46, 33-44.
- 5 Yokoyama, N., Kawano, M.A., Tsukamoto, H., Enomoto, T., Inoue, T., Takahashi, R.U.,
- 6 Nakanishi, A., Imai, T., Wada, T., Handa, H., 2007. Mutational analysis of the
- 7 carboxyl-terminal region of the SV40 major capsid protein VP1. *J. Biochem.* 141, 279-286.
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Figure legends

Fig. 1. Synthesis and characterization of VP1-CMNPs. (a) Schematic diagram of the construction of VP1-CMNPs. SV40-VLPs were dissociated into VP1-pentamers. The surfaces of CMNPs were then coated with VP1-pentamers (VP1-CMNPs). (b) Three different-sized CMNPs of 8, 20, and 27 nm in diameter were incubated with (+) or without (-) VP1^{WT}-pentamers during dialysis. The samples were visualized by negative staining under the TEM. Scale bar: 100 nm. (c) Diameters of intact CMNPs (black bars) and VP1^{WT}-CMNPs (white bars) in the physiological buffer were measured by DSL. Data are shown as the mean $\pm$ SD. (d) Zeta-potential of intact CMNPs, VP1^{WT}-CMNPs, or SV40-VLPs was analyzed. Data are shown as the mean $\pm$ SD. (e) T1-weighted (left) and T2-weighted (right) MR images of MNP (Upper, Ferucarbotran, resovistTM), Gd-DTPA and 27 nm VP1-CMNPs at various concentrations. Samples of distilled water were measured as references.

Fig. 2. Requirements of the pentamer-pentamer interaction in the VP1 coating. (a) VP1^{WT}-CMNPs (left panel) and VP1 ^{Δ C58}-CMNPs (right panel) of 8 nm in diameter were observed under the TEM. Scale bar: 100 nm. (b) Sucrose gradient sedimentation analyses of VP1^{WT}-CMNPs (upper panel) and VP1 ^{Δ C58}-CMNP (lower panel) of 8 nm in diameter

were performed. Fractions were separated by 10% SDS-PAGE and analyzed by Western blot with anti-VP1 antibody. Fractions were numbered from the top to the bottom of the gradient.

Fig. 3. Dispersibility of VP1-CMNPs *in vitro* and *in vivo*. (a) Intact CMNPs (upper panels) and VP1^{WT}-CMNPs (lower panels) of 27 nm in diameter were incubated in water (left panel), PBS (center panels), or FBS (right panels), and were observed under the TEM. Scale bar: 100 nm. (b) Diameters of intact CMNPs and VP1^{WT}-CMNPs in water, PBS, or FBS were analyzed by DLS. (c) BALB/c mice were injected intravenously with either intact CMNPs or VP1^{WT}-CMNPs of 27 nm in diameter. Blood was collected from each mouse at various time points after injection, and the concentration of Fe²⁺ in the blood was measured by ICP-OES analyzer. Data are shown as the mean $\pm$ SD of three samples.

Fig. 4. Targeting of EGFR-overexpressing tumor cells by EGF-conjugated VP1-CMNPs. (a) Schematic diagram of EGF conjugation. EGF was conjugated onto the surface of VP1-CMNPs of 27 nm in diameter using SM(PEG)₂ crosslinker. (b) VP1^{WT}-CMNPs and VP1^{N138C}-CMNPs with (+) or without (-) EGF were loaded onto 10% SDS-PAGE. Positions of EGF conjugated VP1 and free VP1 were indicated. (c) TEM imaging of

1 unconjugated-VP1^{N138C}-CMNPs (left panel) and EGF-conjugated VP1^{N138C}-CMNPs (right
2 panel). Scale bar: 100 nm. (d, e) Cell binding assay. A431 or WiDr cells were incubated
3 with EGF-conjugated either VP1^{WT}-CMNPs or VP1^{N138C}-CMNPs in the presence or
4 absence of various amounts of free EGF. After incubation, cell lysate were separated by
5 10% SDS-PAGE, and analyzed by Western blot with anti-VP1 and anti-actin antibodies (d).
6 Alternatively, samples were subjected to ICP-OES analysis to measure the concentration of
7 Fe²⁺ (e). Data are shown as the mean $\pm$ SD of triplicate samples.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Core diameter	8 nm		20 nm		27 nm	
Coating	(-)	VP1	(-)	VP1	(-)	VP1
r_1 ($s^{-1} mM^{-1}$)	30.1	14.8	29.8	16.5	34.3	20.4
r_2 ($s^{-1} mM^{-1}$)	54.7	71.3	268.1	294.3	317.0	238.0
r_2 / r_1	1.8	4.8	9.0	17.8	9.2	11.7

Table 1: relaxivities for different sized CMNPs with and without VP1-coating