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Modular Bioconjugates to Study Herceptin Resistance: A Structural and Functional Approach

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14. ABSTRACT The focus of the studies described herein was to apply the developed antibody-capsid conjugates to both the examination of ultrastructural features relevant to invadopodia formation as well as mass cytometry probes for the analysis of signaling dynamics in Herceptin resistance development. In working to achieve these goals, a number of challenges were encountered, and the experimental details and assessments are described in the attached report.					
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## 1. Introduction

Understanding the mechanisms by which breast cancers develop resistance to therapy is an important and active area of research. Breakthroughs in this area have the potential to identify unforeseen targets for therapy, with the potential to even develop synthetic lethality through targeting multiple pathways at once. Resistance to Herceptin therapy represents a particularly troubling case due to the high number of breast cancers that overexpress the HER2 receptor. The overall goal of the present work is directed toward understanding structural and functional changes that take place within breast cancer cells that leads to the observed resistance phenotype. In order to study this system, a collection of modular bioconjugates will be synthesized that will allow in depth profiling of the relevant pathways. In this reporting period, the major focus has been working to apply the conjugates described in the previous reporting period to the structural and functional studies of Herceptin resistance.

## 2. Keywords

bioconjugation, oxidative coupling, viral capsids, antibodies, HER2, EGFR, mass cytometry, fluorescence microscopy

## 3. Accomplishments

### Major Goals of Project:

Below are the stated tasks in the approved statement of work. Text in bold+italics provides information on the progress relative to the stated goals and/or milestones.

#### **Task 1. Preparation and validation of targeted, modular mAb-MS2 bioconjugates (months 1-36 as needed throughout the duration of the project):**

1a. Refinement of protocol for attachment of mAb to MS2 capsids (months 1-6, **completed**).

1b. Evaluation of the specificity of these conjugates by flow/mass cytometry, confocal microscopy, and electron microscopy (months 6-12 and as needed as new targets are explored and need validation, **characterization and validation completed for HER2 and EGFR which were used for proof of concept**).

*Milestone(s) Achieved: Preparation and validation of the conjugates to be used for the ensuing studies. Publication of these results in a peer-reviewed journal (12 months, completed and disclosed in previous period report).*

#### **Task 2. The study of morphological changes with respect to invadopodia formation in cells upon treatment with Herceptin (months 12-30):**

1a. TEM studies of healthy cells and tumorigenic cells with conjugates described in Task 1 (months 6-18, **5% completed**).

1b. iLEM studies of tumorigenic cells that have been cultured in 2D and 3D in response to prolonged Herceptin exposure (months 18-30, **0% completed**).

*Milestone(s) Achieved: A full assessment of the role, if any, that invadopodia structures play in Herceptin resistance mechanisms (30 months, **0% completed**). Publication of results in peer-reviewed journals (36 months).*



**Task 3. The study of signaling dynamics in HER2-overexpressing breast cancer cells in response to various clinically relevant therapeutics (months 12-36):**

1a. Development of a signaling fingerprint of >10 breast cancer cell lines using cyTOF before treatment with Herceptin and other clinically relevant therapies (months 6-18, **5% completed**).

1b. CyTOF studies of the signaling dynamics that change upon initial and prolonged treatment of the aforementioned cell lines with clinically relevant therapies (months 18-30, **0% completed**).

1c. In-depth data analysis to uncover patterns arising in the results (months 6-36, **0% completed**).

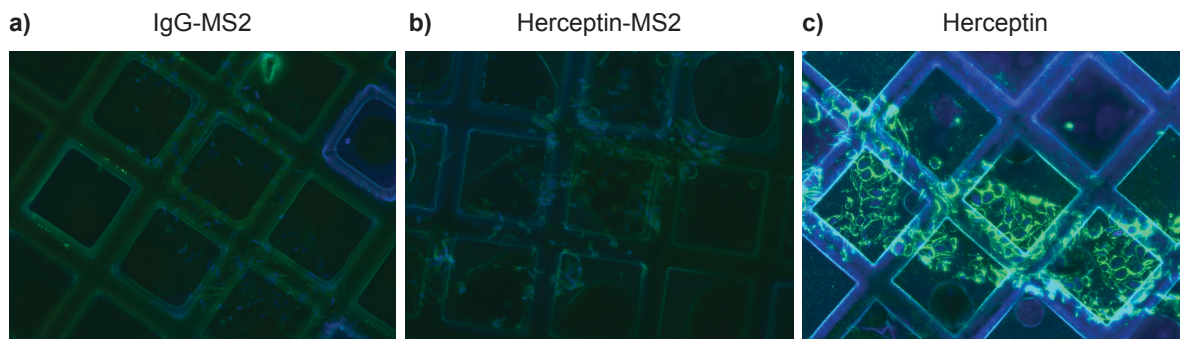
*Milestone(s) Achieved: Preparation of cyTOF agents for detection of ultra-low abundance analytes (24 months). Network analysis of the signaling dynamics in breast cancer cells in response to Herceptin treatment (30 months, **0% completed**).*

*Publication of results in peer-reviewed journals (36 months).*

**Accomplishments Relative to Goals:**

For this period of reporting, experimental effort was divided between Tasks 2a and 3a. In both cases, extensive challenges were encountered in applying the technology developed during the first reporting period to these tasks, and the efforts in this regard are described below and summarized briefly in Section 5.

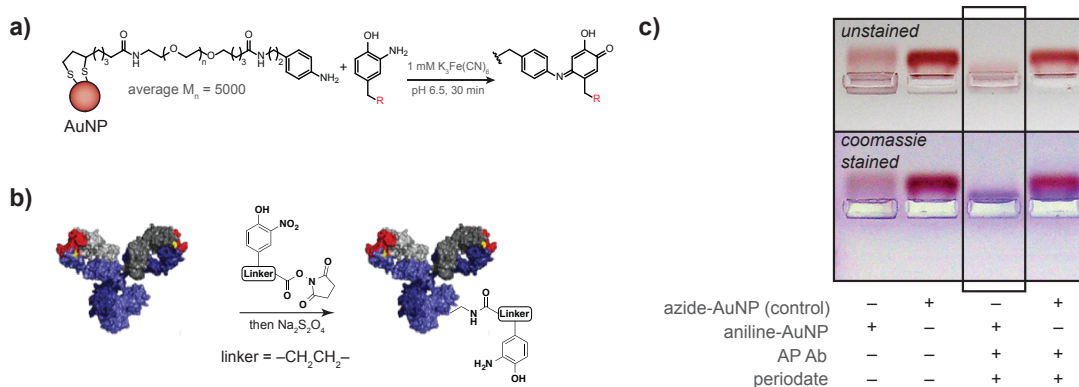
With regards to Task 2, the first step in establishing the morphological changes in invadopodia formation relies on an ability to stain fixed cells for application in both fluorescence and TEM imaging. A technical requirement for these applications is that the cells must be embedded in a matrix and fixed prior to cell staining and image acquisition. This task required me to learn and optimize this protocol for embedment of SKBR3 cells in matrigel, and the Tokuyasu sectioning techniques were optimized by members of Prof. Joe Gray's group at OHSU. The latter task requires a significant amount of experience to ensure that ultrastructural features, such as invadopodia, are retained during the sectioning. With these tasks completed, capsid agents bearing humanized anti-HER2 antibodies (i.e. Herceptin) on their exterior surface and Oregon Green 488 fluorophores on the interior surface were sent to OHSU for analysis on Tokuyasu sections of SKBR3 cells embedded and fixed in matrigel. Drs. Danielle Jorgens and Anke Mulder used these staining reagents along with a negative control reagent that had an untargeted, random IgG on the capsid surface and fluorescently labeled Herceptin (positive control) in a cell-staining experiment. Unfortunately, under a variety of conditions, only very weak cell staining was observed with the Herceptin-targeted capsid reagents while the Herceptin positive control reagents stained the cell surfaces quite well. In order to determine if the capsid targeting ability had been affected during the sample shipping, the agents were used in a quick flow cytometry experiment on SKBR3 cells, and this experiment demonstrated good binding. Several other experiments with slightly altered protocols also failed to exhibit efficient cell surface staining with our reagents, and representative examples of these experiments are provided in Figure 1. Our current hypothesis regarding the discrepancy between the isolated flow cytometry experiments and the Tokuyasu section staining is that the capsids may be too large in diameter to efficiently penetrate the pores of the crosslinked 3D matrix.



**Figure 1.** Cell staining of SKBR3 cells prepared from Tokuyasu sections. Cells were fixed and embedded in matrigel then treated with **a)** random IgG conjugated to OregonGreen 488 fluorescent MS2 capsids, **b)** Herceptin-targeted OregonGreen 488 MS2 capsids, or **c)** AlexaFluor 488-conjugated Herceptin. While higher levels of staining are observed in Herceptin-MS2 compared to IgG-MS2, these levels of staining are much less efficient than that of Herceptin alone.

With regards to task 3, the proteins relevant for the signal transduction cascade process are cytosolic in nature, which requires that the cells be permeabilized and fixed during the staining process. In our previous mass cytometry staining protocol with capsid agents disclosed in the first reporting period, live cells were used for the staining protocol, and extensive optimization was required to achieve efficient staining with low background. In order to adapt this protocol, we elected to examine the use of anti-HER2-bearing capsids with the new staining protocol (i.e. with fixation and permeabilization) to determine if low background could be maintained. In this case, the permeabilization protocols, which have significant levels of methanol and para formaldehyde in the solution, caused the viral capsid to disassemble, leading to very high levels of background due to non-specific sticking/crosslinking of the capsid proteins to cellular proteins. The background labeling was confirmed by the use of untargeted capsids, which showed high signal in the presence of fixation protocols and nearly undetectable levels when staining live cells. Despite many efforts to chemically stabilize the capsid to prevent disassembly, I was unable to identify a method that would prevent its disassembly.

The difficulties described above led us to the conclusion that the capsid bioconjugates may not prove capable of application to tasks 2 and 3, thus we required a method to achieve similar results by conjugation directly to antibodies of choice. This is particularly important in the case of task 2 where so-called immunogold reagents are not available in any format for many of the proteins relevant to the signaling network of interest. In 2014, we reported an efficient method for the conjugation of gold nanoparticles to biomolecules, such as DNA and proteins, by use of a novel ligand framework and a mild oxidative coupling protocol (manuscript attached as Appendix B). Here, we hoped that we may be able to adapt our protocol for attaching antibodies to the surface of the MS2 viral capsid to the attachment to gold nanoparticles. With some optimization, this goal has been achieved, as depicted in Figure 2. In particular, we have recently found that periodate-mediated oxidative coupling anti-HER2 antibodies to gold nanoparticles was successful, as determined by native agarose gel electrophoresis. Thus, efforts towards tasks 2 and 3 can recommence.



**Figure 2.** Optimization of a protocol for labeling gold nanoparticles with antibodies. **a)** Gold nanoparticles capped with lipoamide-PEG-aniline ligands (aniline-AuNP) are efficient in conjugation of biomolecules bearing aminophenols in a mild oxidative coupling procedure (see Appendix B for full details). **b)** Antibodies are labeled with an NHS/nitrophenol heterobifunctional linker with subsequent chemoselective dithionite reduction to yield antibody-aminophenol conjugates (AP Ab). **c)** Conjugation of the AP Ab to gold nanoparticles in the presence of periodate is only observed when aniline ligands are present on the gold nanoparticles, as demonstrated by the fact that the AuNPs and antibodies show the same electrophoretic mobility only when the aniline ligands and aminophenols are present in the presence of oxidant (lane 3). Negative control azide gold nanoparticles show no conjugation (lane 4).

## Training/Professional Development:

During the training period, the professional developments described below were undertaken:

### Conferences/Workshops:

- Attended the Gordon Research Conference in Bioorganic Chemistry
- Participated in the genetic code expansion workshop at Oregon State University

## Dissemination to Communities of Interest:

Nothing to report

## Plans for Next Reporting Period:

None. This is a final annual report.

## 4. Impact

### Principal Discipline:

A new technique for conjugating antibodies to gold nanoparticles was developed which may find widespread adoption for its synthetic ease as well as generality.

### Other Disciplines:

None to report.

### Technology Transfer:

Nothing to report.

### Greater Society:

Nothing to report.

## 5. Changes/Problems

### Changes in Approach:

Unfortunately, the capsid conjugation strategy appears to be incompatible with the two major techniques described in Tasks 2 and 3, and therefore, we have begun to develop direct antibody conjugates to achieve the goals outlined in those tasks.

### Problems/Delays and Resolutions:

Although the agents prepared during the last reporting period demonstrated highly efficient binding to cells in flow cytometry experiments as well as live-cell confocal microscopy studies, our ability to stain Tokuyasu sections from cells embedded in matrigel was much less efficient for reasons that are still unclear. Furthermore, the capsid conjugates were found to be incompatible with the fixation and permeabilization protocols for mass cytometry. In this case, it appears the capsids disassemble under the conditions typically used for these protocols, leading to high background signal in these experiments.

In order to begin to address these issues, we have moved away from the capsid conjugates and are working to develop direct antibody conjugates for both TEM visualization and mass cytometry experiments.

The major delay in the present reporting period was due to a personal illness that precluded my ability to be in the laboratory for an extended period of time.

### Changes Impacting Expenditures:

Nothing to report.

## 6. Products

### Journal Publications:

- I. L. Aanei, A. M. ElSohly, M. E. Farkas, C. Netirojjanakul, M. Regan, S. Taylor Murphy, J. P. O'Neil, Y. Seo, and M. B. Francis. Biodistribution of Antibody-MS2 Viral Capsid Conjugates in Breast Cancer Models. *Mol. Pharm.* **2016**, *in press*. Published with acknowledgement of federal support and attached as Appendix A.

## 7. Participants & Other Collaborating Organizations

### Individuals:

Name:	Adel ElSohly
Project Role:	Postdoc (PI)
Person Months Worked:	12
Contribution to Project:	Conducted all aspects of research, including design and execution of experiments
Funding Support	This grant (BC134050)

Name:	Ioana Aanei
Project Role:	Graduate Student
Person Months Worked:	2
Contribution to Project:	Assisted in optimization of mass cytometry labeling protocol
Funding Support	Genentech graduate fellowship

**Changes in Active Other Support:**

Nothing to report.

**Other Organizations:**

Organization Name:	Stanford University
Location:	Paolo Alto, CA (USA)
Organization Contribution:	Facilities

**8. Special Reporting Requirements**

Nothing to report

**9. Appendices**

Appendix A: Preprint of “Biodistribution of Antibody-MS2 Viral Capsid Conjugates in Breast Cancer Models.”

Appendix B: Reprint of “Bioconjugation of Gold Nanoparticles through the Oxidative Coupling of ortho-Aminophenols and Anilines.”

# Biodistribution of Antibody-MS2 Viral Capsid Conjugates in Breast Cancer Models

Ioana L. Aanei,<sup>†,‡</sup> Adel M. ElSohly,<sup>†</sup> Michelle E. Farkas,<sup>†</sup> Chawita Netirojjanakul,<sup>†</sup> Melanie Regan,<sup>§</sup> Stephanie Taylor Murphy,<sup>§</sup> James P. O'Neil,<sup>‡</sup> Youngho Seo,<sup>§</sup> and Matthew B. Francis<sup>\*,†,‡</sup>

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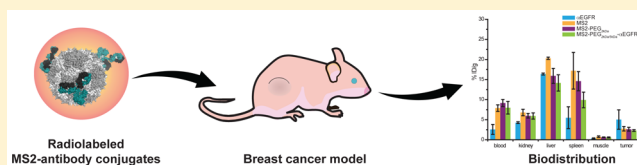
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## Supporting Information

**ABSTRACT:** A variety of nanoscale scaffolds, including virus-like particles (VLPs), are being developed for biomedical applications; however, little information is available about their *in vivo* behavior. Targeted nanoparticles are particularly valuable as diagnostic and therapeutic carriers because they can increase the signal-to-background ratio of imaging agents, improve the efficacy of drugs, and reduce adverse effects by

concentrating the therapeutic molecule in the region of interest. The genome-free capsid of bacteriophage MS2 has several features that make it well-suited for use in delivery applications, such as facile production and modification, the ability to display multiple copies of targeting ligands, and the capacity to deliver large payloads. Anti-EGFR antibodies were conjugated to MS2 capsids to construct nanoparticles targeted toward receptors overexpressed on breast cancer cells. The MS2 agents showed good stability in physiological conditions up to 2 days and specific binding to the targeted receptors in *in vitro* experiments. Capsids radiolabeled with <sup>64</sup>Cu isotopes were injected into mice possessing tumor xenografts, and both positron emission tomography-computed tomography (PET/CT) and scintillation counting of the organs *ex vivo* were used to determine the localization of the agents. The capsids exhibit surprisingly long circulation times (10–15% ID/g in blood at 24 h) and moderate tumor uptake (2–5% ID/g). However, the targeting antibodies did not lead to increased uptake *in vivo* despite *in vitro* enhancements, suggesting that extravasation is a limiting factor for delivery to tumors by these particles.

**KEYWORDS:** bacteriophage MS2, tumor targeting, antibody targeting, bioconjugation, PET imaging



## INTRODUCTION

Despite numerous advances over the past several decades, the effective detection and treatment of cancer remain challenging.<sup>1</sup> The toxicity of many antitumor drugs makes achieving therapeutic concentrations without severe systemic side effects very difficult.<sup>2</sup> To address these issues, significant efforts have been dedicated to the design of carriers that can deliver the desired cargo selectively to tumor sites. Synthetic platforms, such as polymers,<sup>3</sup> dendrimers,<sup>4,5</sup> liposomes,<sup>6,7</sup> and the protein cage architectures of plant viruses<sup>8,9</sup> and bacteriophages,<sup>10</sup> have been investigated for their potential to serve as *in vivo* delivery vehicles. Most of these vehicles rely on the size of the nanoparticles to take advantage of the increased permeability of the vasculature in the tumor vicinity relative to the rest of the circulatory system to promote passive accumulation.<sup>11</sup> This phenomenon, referred to as the enhanced permeability and retention (EPR) effect,<sup>12–14</sup> is attributed to large spaces between endothelial cells in blood vessels formed during tumor growth<sup>15</sup> and poor drainage by tumor lymphatic systems.<sup>16</sup> Passive targeting of nanoparticles in the 10–100 nm size range has been shown to result in increased tumor

accumulation when compared to small molecules.<sup>13</sup> However, there are numerous challenges in generating monodisperse nanoparticles with controlled size in this size range, which is believed to be ideal for tumor uptake via EPR.<sup>13,17</sup> Different types of targeting groups (small molecules,<sup>18,19</sup> aptamers,<sup>20</sup> peptides,<sup>21,22</sup> glycans,<sup>23</sup> antibodies<sup>24–27</sup>) have been used to decorate nanoparticles with the hope of increasing their homing to the tumor. Among these moieties, antibodies have the advantages of widespread availability, as well as high specificity and affinity for their targets. Liposomes that are decorated with antibodies (immunoliposomes) are some of the most advanced nanoparticles on the way to clinical applications and establish the potential of antibodies to be used as targeting moieties on nanoscale carriers *in vivo*.<sup>28,29</sup>

Protein cages have also drawn attention for their ability to carry increased levels of cargo molecules.<sup>29</sup> In addition, protein-

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based delivery agents have several advantages over polymer-based platforms, such as homogeneity, biocompatibility, and biodegradability.<sup>30</sup> Although great strides are being made in the tailoring of the *in vivo* properties of protein carriers, most nanoparticles still suffer from rapid clearance from the body due to the reticuloendothelial system (RES) comprising the liver and spleen.<sup>31,32</sup> For example, upon intravenous administration of the Cowpea Mosaic Virus (CPMV) to mouse models,<sup>33,34</sup> the particles localize mainly in the liver and the spleen, are rapidly cleared from plasma, and reach undetectable levels within 20 min. Similarly, the Potato Virus X (PVX) has a short half-life *in vivo* of only 12.5 min.<sup>35</sup> The Cowpea Chlorotic Mottle Virus (CCMV) and Heat Shock Protein (Hsp) also exhibit short circulation times, with less than 1% of the injected dose per gram (% ID/g) still present in the mouse blood at 24 h postinjection.<sup>36</sup>

Recent studies suggest that modification of the surface of nanoparticles with passivating layers, such as polyethylene glycol (PEG), can delay systemic clearance, thus allowing for increased tumor homing.<sup>37</sup> The immunogenicity of viral capsids could also be mitigated by PEGylation of virus-like particle (VLP) constructs, based on studies showing that antibody recognition of the viral capsid proteins can be decreased by PEG chains.<sup>38–41</sup> However, additional research is required to develop a comprehensive understanding of VLP behavior *in vivo*, including biodistribution, clearance rates, immunogenicity, and toxicology, in the interest of facilitating the translation of virus capsid-based nanomedicines from the research laboratory to the clinic.<sup>42</sup>

Herein, we describe a targeted nanoscale imaging platform based on bacteriophage MS2, the VLPs of which self-assemble from 180 copies of a single coat protein (13.8 kDa) into monodisperse, 27 nm icosahedral capsids. MS2 capsids are biodegradable, stable under a variety of temperature, pH, and solvent conditions, and easily synthesized and purified in large quantities.<sup>21</sup> Recently, our group has designed orthogonal chemistries to allow the exterior and interior surfaces to be modified independently.<sup>21,43–46</sup> Through judicious use of these reactions, the viral capsid can house multiple copies of a given drug molecule or imaging agent, potentially offering a significant increase in therapeutic index<sup>20,45,47</sup> or signal intensity,<sup>48,49</sup> respectively. The capsid exterior has also been endowed with cell-specific targeting capabilities via the appendage of receptor-specific antibodies on the external surface. Given the potential of MS2 as a drug delivery and imaging agent, and its success in *in vitro* experiments,<sup>46,50</sup> the *in vivo* behavior of constructs based on this scaffold warrants investigation. The study presented herein therefore lays the foundation for the development of the MS2 bacteriophage into a tailorable delivery system for many future applications.

## MATERIALS AND METHODS

**Materials.** Unless otherwise noted, all chemicals and solvents were of analytical grade and used as received from commercial sources. Anti-EGFR human IgG1 monoclonal antibody was obtained from Eureka Therapeutics, Inc. (Emeryville, CA). All polyethylene glycol (PEG) derivatives were purchased from Laysan Bio, Inc. (Arab, AL). All cell culture reagents, including normal mouse serum, were obtained from Gibco/Thermo Fisher (Waltham, MA) unless otherwise noted. Maleimide-DOTA (1,4,7,10-tetraazacyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide) and pSCN-Bn-NOTA (2-S-(4-isothiocyantobenzyl)-1,4,7-triazacyclono-

nane-1,4,7-triacetic acid) were purchased from Macrocylics (Dallas, TX). Maleimide-NOTA (2,2'-(7-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-2-oxoethyl)-1,4,7-triazonane-1,4-diyl)diacetic acid), maleimide-NOTA-GA (2,2'-(7-(1-carboxy-4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diacetic acid), and maleimide-DOTA-GA (2,2',2''-(10-(1-carboxy-4-((2-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)ethyl)amino)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) were purchased from CheMatech (Dijon, France). Ethylenediaminetetraacetic acid (EDTA) disodium salt and potassium phosphate dibasic were purchased from EMD Chemicals Inc. (Darmstadt, Germany). Copper-64 was purchased from the Medical Cyclotron Laboratory, University of Wisconsin. Saline (sodium chloride) solution was Injection USP, 0.9% from APP Pharmaceuticals (Schaumburg, IL).

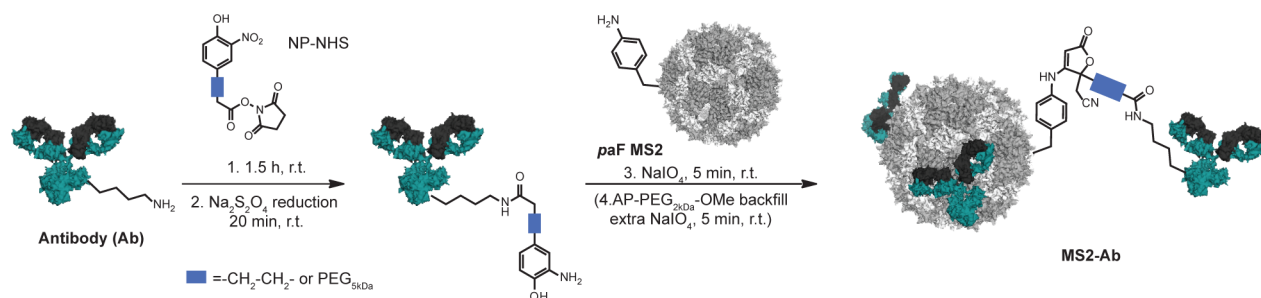
**Methods. Synthesis and Characterization of MS2–Ab Conjugates.** The protocol for constructing MS2–Ab conjugates was described recently.<sup>46</sup> For full details, please see the [Supporting Information](#).

**Immunogenicity Assays.** In order to investigate the immune response that the capsids might trigger in a living system, we contracted an outside company, Pacific Bio Laboratories, to test the amount of antibody produced upon tail vein injections of MS2 conjugates. Mice with intact immune systems (female Balb/c, 3 mice per group) were injected intravenously (iv) with 100  $\mu$ L of agent in sterile saline at  $t = 0$  and with a second dose at 3 weeks. Blood samples were obtained before the first injection and at 2, 4, and 8 weeks. The amount of antibody present was determined using commercially available ELISA kits for IgM and IgG quantification. The experimental procedure details are described in the [Supporting Information](#).

**Radiolabeling of MS2 Conjugates.** To a 100  $\mu$ M sample of protein (based on capsid monomer) in 10 mM potassium phosphate buffer, pH 7.2, was added 10 equiv of maleimide-chelator from a 10 mM stock in DMSO. The reaction was allowed to proceed for 4 h at room temperature and was purified using a Nap-10 Sephadex size exclusion column (GE Healthcare, Pittsburgh, PA) equilibrated with phosphate buffer, pH 6.5 and 100 kDa molecular weight cutoff (MWCO) spin concentrators (Millipore, Billerica, MA). For the Ab-NOTA samples, the antibodies were first reacted with 200 equiv of pSCN-Bn-NOTA at 37 °C for 24 h. The reaction was stopped by using a Nap-5 desalting column, and the purified antibody was concentrated to 10–20  $\mu$ M using a 30 kDa spin concentrator (Millipore).

The <sup>64</sup>Cu copper stock (20–30 mCi, ~200  $\mu$ L) was diluted with 1 mL of 0.1 M ammonium citrate buffer, pH 6.2 to generate a final volume of ~1200  $\mu$ L at pH 5.5 (determined by pH paper). Each reaction tube was then charged with 100–400  $\mu$ L of diluted <sup>64</sup>Cu solution and 50–150  $\mu$ L of the MS2 conjugates (200  $\mu$ M in capsid monomer). The complexation reactions were allowed to proceed for 2 h at room temperature and then purified using Nap-5 or Nap-10 columns. Samples were subsequently concentrated using 100 kDa MWCO spin concentrators. Centrifugation was performed at 10,000 rpm for 5 min per round of concentrating until the desired volume was reached.

**In Vitro Stability Studies.** Concentrated <sup>64</sup>Cu-chelator-MS2 conjugates (~300  $\mu$ Ci in 20  $\mu$ L) were added to PBS or 100% mouse serum to a final volume of 200  $\mu$ L (1:9 v/v). The samples were then incubated at 37 °C for a predetermined time using a temperature-controlled heat block (VWR, Radnor, PA).



**Figure 1.** Generation of MS2–antibody conjugates. Nitrophenol (NP) groups were attached to antibodies (Ab) via lysine modification using NP-NHS. The nitrophenol groups were then reduced to yield aminophenol–antibody conjugates (AP–Ab) by addition of  $\text{Na}_2\text{S}_2\text{O}_4$ . The resulting AP–Ab were coupled to *p*-aminophenylalanine (pF) MS2 via an oxidative coupling using  $\text{NaIO}_4$  as oxidant.

Aliquots were drawn from the samples at 1, 4, 8, 24, and 48 h time points, and injected onto a PolySep GFC-P5000 (Phenomenex, Torrance, CA) size exclusion chromatography column ( $300 \times 7.8$  mm,  $5 \mu\text{m}$  particle size,  $500 \text{ \AA}$  pore size; column flow rate  $1.5 \text{ mL/min}$  of  $10 \text{ mM KH}_2\text{PO}_4$  containing  $1 \text{ mM}$  disodium EDTA, pH 7.2). The HPLC system consisted of a 590 HPLC pump (Waters, Milford, MA), UV detector operating at  $280 \text{ nm}$  (Linear Systems, Fremont, CA), model 105S-1 high-sensitivity radiation detector with  $1 \text{ cm}^3$  CsI (T1) scintillating crystal coupled to a  $1 \text{ cm}^2$  Si PIN photodiode/low-noise preamplifier (Carroll-Ramsey Associates, Berkeley, CA), and fluorescence detector (Spectra system FL3000, Thermo Separation Products, St. Peters, MO). Chromatography traces were collected using PeakSimple data system and software (SRI Instruments, Torrance, CA), and analyzed using the Gaussian multippeak fitting feature of the OriginPro software v. 8.6.0 (OriginLab, Northampton, MA).

**PET/CT and Biodistribution Studies.** All animal procedures were performed according to a protocol approved by the UCSF Institutional Animal Care and Use Committee (IACUC). Six-week-old female nu/nu (nude) mice weighing  $18\text{--}23 \text{ g}$  were purchased from Charles River Laboratories (Hollister, CA). For tumor inoculation, the cells were implanted in the number 4 mammary fat pad ( $\beta$ -estradiol pellets were also implanted subcutaneously in the flank for MCF7 clone 18 cells) or in the right flank for subcutaneous models. The imaging and biodistribution experiments were started approximately 2 weeks following implantation, when the tumors were  $\sim 1 \text{ cm}$  in diameter.

Tumor-bearing nude mice in sets of 3 animals per study group were injected in the tail vein (intravenous, iv) with  $150\text{--}250 \mu\text{Ci}$  ( $5.5\text{--}9.25 \text{ MBq}$ ) of  $^{64}\text{Cu}$ -labeled MS2 conjugates in  $100 \mu\text{L}$  of sterile saline. One animal from each group was selected for imaging with microPET/CT (Inveon microPET docked with microCT, Siemens Molecular Solutions, Malvern, PA). The list mode data were acquired from the time of injection to 1 h postinjection, and dynamic multiframe reconstruction was performed to capture dynamics of  $^{64}\text{Cu}$ -labeled MS2 conjugates. In addition, a 20 min static scan was performed at 24 h postinjection. CT scans were performed after each PET scan to provide anatomical localization of radionuclide data, as well as photon attenuation map for attenuation-corrected PET reconstruction. Images were reconstructed using the AMIDE software v.1.0.4.

The dynamic images were used for determining the circulation profile of the agents by integrating the signal over a region of interest in the left ventricle of the heart (AMIDE

software). The  $\log_{10}$  of the integrated signal was plotted versus the midpoint of each scan frame.

Mice were sacrificed at 1 or 24 h postinjection, and the blood, tumor, and major organs were harvested and weighed. The radioactivity present in each sample was measured using a Wizard gamma counter (PerkinElmer, Waltham, MA). All values were decay corrected, and the percentage injected dose per gram (% ID/g) was calculated for each tissue sample. Means and standard deviations were calculated for each group. Using Excel software (Microsoft, Redmond, WA), an unpaired *t* test with equal variance and a two-tailed *p* value was performed for organs from different data sets. A result was considered statistically significant if it occurred at the  $p < 0.05$  level.

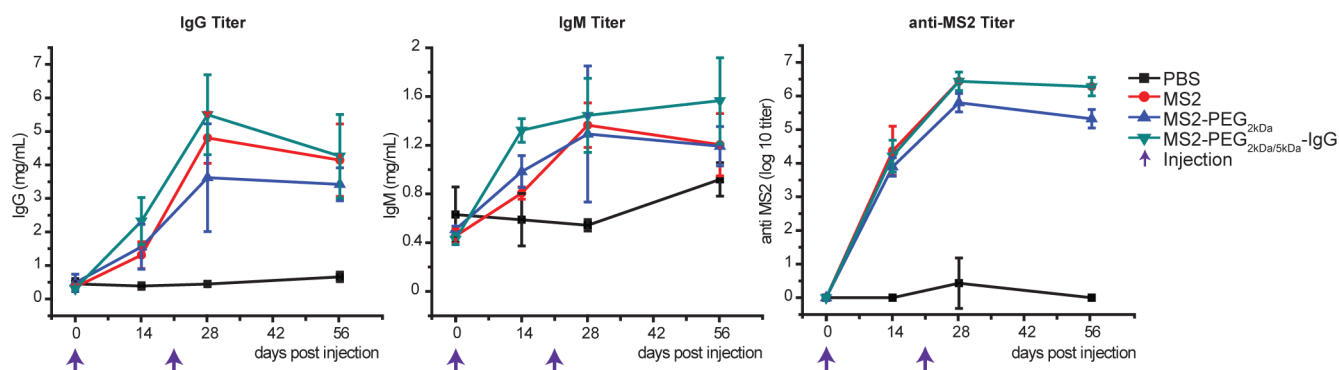
To determine the assembly state of the agents after circulating *in vivo*, blood samples were allowed to coagulate (30 min at room temperature) and serum was collected by spinning at  $10000g$  for 10 min and removing the supernatant. Serum aliquots were injected into an SEC HPLC system and the radioactivity channel was monitored for peaks corresponding to the assembled MS2 capsid, its monomer units, or  $^{64}\text{Cu}$  associated with other serum proteins.

## RESULTS AND DISCUSSION

**Synthesis and Characterization of MS2–Antibody Conjugates.** We first synthesized a series of MS2–antibody (MS2–Ab) conjugates (Figure 1, Supplementary Figure S1) using a recently developed oxidative coupling strategy for conjugating two macromolecules in a rapid and high-yielding reaction.<sup>46</sup> Control over the site for antibody attachment was attained by introducing the *p*-aminophenylalanine (pF) unnatural amino acid at position 19 of the MS2 capsid proteins.<sup>51,52</sup> This residue is located on the exterior surface of the assembly and reacts readily with aminophenol groups in the presence of mild oxidants, as shown previously.<sup>21,44,46</sup> For achieving targeting, we used a humanized monoclonal antibody against the epidermal growth factor receptor ( $\alpha$ -EGFR), although similar results were observed with the anti-human epidermal growth factor receptor ( $\alpha$ -HER2) antibody (data not shown). The EGFR and HER2 receptors are targets of interest since they are overexpressed in numerous types of cancer, such as breast cancer, glioma, and colon cancer.<sup>53–55</sup>

We explored the use of linkers between the viral capsids and the antibodies (Figure 1) to allow for efficient interaction of the antibody with the receptor targeted. Flow cytometry experiments (*vide infra*, Supplementary Figure S2) suggest that the length of the linker (two carbon atoms, or polyethylene glycol with molecular weights of  $2 \text{ kDa}$  or  $5 \text{ kDa}$ ) did not affect the affinity of the antibody to its target. The PEG chain, however,





**Figure 2.** Immunogenicity assay. The amount of IgG, IgM, and anti-MS2 coat protein antibodies in the serum of Balb/c mice was measured at different time points postinjection using ELISA assays. Although the amount of antibodies produced for all of the MS2 samples was statistically higher than the PBS negative control (black line), the differences between the agents were not significant (unpaired *t* test with equal variance and a two-tailed *p* value, *p* > 0.05).

could have a shielding effect from the immune system and therefore was used in most *in vivo* experiments. We also explored constructs with a small number of antibodies linked to the MS2 capsids through PEG<sub>5 kDa</sub> linkers and additional surface coating with PEG<sub>2 kDa</sub> (i.e., “backfilling” via a subsequent oxidative coupling reaction) in order to shield the surface of the viral capsid while still allowing the antibodies to interact unobstructedly with their targets.<sup>56</sup>

Dynamic light scattering (DLS) and transmission electron microscopy (TEM) studies (Supplementary Figure S1) suggested homogeneous populations of MS2 derivatives, with the hydrodynamic radii increasing from 27 to 30.7 nm upon conjugation of antibodies, and to 28.9 nm when PEG<sub>5 kDa</sub> was used as a linker. In the backfilling case, the measured diameter was 33.7 nm. We hypothesize that, when the two carbon linker is used, the antibodies are attached tangentially to the capsid surface, explaining the relatively small increase in diameter. Given the surface of the MS2 bacteriophage (~2300 nm<sup>2</sup>) and the number of PEG chains attached (100–130 per capsid), the density of PEG chains is likely insufficient to extend the polymers to the “brush” conformations.<sup>57,58</sup> This expectation is supported by the fact that the predicted length of the fully extended PEG chain is ~16 nm for PEG<sub>2 kDa</sub> and ~35 nm for PEG<sub>5 kDa</sub>,<sup>59</sup> but only small increases in nanoparticle diameter are observed by DLS.

**In Vitro Interactions with Cancer Cell Models.** Fluorescently labeled MS2 conjugates were used in flow cytometry and confocal microscopy experiments to investigate their interaction with breast cancer cell lines. These samples were prepared by reacting cysteine residues introduced at position 87 with Oregon Green 488 (OG488) maleimide dyes, as shown in Supplementary Figure S2a. Mass spectrometry analysis shows that near-complete modifications can be obtained, resulting in agents with more than 100 copies of the small molecule attached. Subsequent incubation of these samples with two breast cancer cell lines indicated that the targeted agents have specific interactions with surface receptors of interest (Supplementary Figure S2b). The addition of PEG linkers to extend the antibody further from the capsid surface did not seem to improve the binding to the targeted receptor (Supplementary Figure S2c). Flow cytometry experiments (Supplementary Figure S2d) did not reveal any clear advantage of having more than 3 copies of the antibody per capsid, and thus for cost efficiency, conjugates with three or fewer copies of antibodies were used for all subsequent studies. Preincubation

of the agents in mouse serum did not inhibit their ability to bind the cells (Supplementary Figure S2e), suggesting that serum proteins are not detrimental to the binding of the MS2–Ab conjugates.<sup>56</sup>

Live-cell confocal microscopy with these agents confirms that the binding is specific to the cells expressing the targeted receptor (Supplementary Figure S3). HCC1954 cells were incubated for 1 h at 37 °C with OG488-labeled MS2, MS2–αEGFR, and MS2-PEG<sub>5 kDa</sub>-αEGFR in DPBS with 1% FBS. Fluorescent signal was observed on the cell surfaces and, at later time points, inside the cells, in several vesicles. These results indicate that, upon binding to the surface receptors, the constructs are internalized.

**In Vivo Assessment of Immunogenicity of MS2–Ab Conjugates.** Balb/c mice with intact immune systems were administered MS2-based agents through tail vein injection in two doses, at *t* = 0 and 3 weeks. The agents tested were MS2, MS2 coated with ~130 strands of methoxy-terminated PEG<sub>2 kDa</sub> (MS2-PEG<sub>2 kDa</sub>), and MS2 decorated with both antibodies with PEG<sub>5 kDa</sub> spacers and PEG<sub>2 kDa</sub> backfilling (MS2-PEG<sub>2 kDa/5 kDa</sub>-IgG). To facilitate the ability to distinguish the immune response caused by the capsids from the immunogenicity of the humanized anti-EGFR antibody, a mouse IgG antibody was used for this study.

After the first dose, the amount of total IgM and IgG produced by animal groups treated with the three agents was similar (0.5–2 mg/mL). Upon injection of the second dose, the amount of IgG produced was 3–4 times higher than the IgM (Figure 2). Brown et al.<sup>24</sup> observed a similar ratio of IgG to IgM antibodies, indicating that, upon multiple administrations of MS2 (albeit subcutaneous in their case), the bulk of the response was in the form of IgG.

Anti-MS2 coat protein antibodies were present in the serum after the initial dosing, and a small increase was observed after the second dose (Figure 2). Using a two sample unpaired Student *t* test, we determined that the amounts of antibodies produced upon injection of the MS2 agents were significantly higher (*p* < 0.05) than the PBS control. The addition of PEG chains did not seem to have a significant effect on the amount of antibody produced. This phenomenon might be explained by the fact that even if there are about 130 strands of PEG per capsid, they might not reach the “brush regime”<sup>57</sup> and could be less efficient at shielding the viral capsid from the immune system.<sup>39</sup> Mastico et al.<sup>60</sup> have also observed the production of anti-MS2 antibodies in mice, which was amplified by the

addition of a nonapeptide derived from hemagglutinin. Although there is a paucity of studies on the immune response caused by iv administration of viral capsids as delivery agents (and not as vaccines), a study by Kaiser et al.<sup>36</sup> noted that antibody production against CCMV and Hsp was also observed.

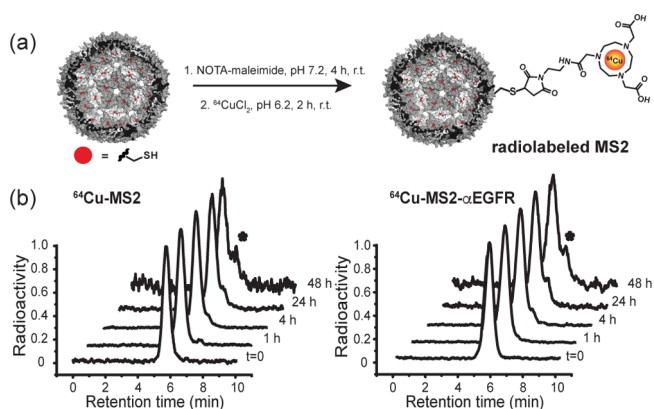
**Radiolabeling of MS2 Capsids.** In biomedical imaging applications based on radioactivity, the radionuclide itself is detected rather than the nanoparticle or the payload. Therefore, the radiolabeling strategy must involve a stable interaction between the tracer and the carrier. Additionally, the radionuclide half-life has to be compatible with the *in vivo* pharmacokinetics of the probe. In recent years, copper-64 (<sup>64</sup>Cu) has attracted increasing attention as a promising radionuclide due to its decay characteristics (half-life  $t_{1/2}$  = 12.7 h) that are compatible with the time scales required for the biodistribution of agents, such as monoclonal antibodies and nanoparticles that exhibit long circulation times. Radiolabeling of systems of interest can be obtained by coordination with ligands such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 2,2',2''-(1,4,7-triazonane-1,4,7-triyl)-triacetic acid (NOTA). These ligands utilize both the macrocyclic and chelate effects to enhance the stability of the chelator–isotope construct.<sup>61</sup>

The radiolabeling of MS2 conjugates was accomplished by incubating the <sup>64</sup>Cu in its chloride form with MS2 capsids modified by reacting chelators (*vide infra*) bearing a maleimide moiety with internal cysteine residues. Up to 180 copies of the chelators were installed on the interior surface of each capsid, leading to an increased load of isotopes inside the VLP (Supplementary Figure S4). Housing the radioisotope inside the capsid also mitigates the ability of the <sup>64</sup>Cu to interact with serum proteins and escape from the carrier. Upon desalting and concentrating steps to remove excess radioisotope, radiochemical yields between 65 and 85% were obtained. The total synthesis time was between 3 and 4 h, leading to >95% pure radiolabeled capsids, as verified by high performance liquid chromatography size exclusion chromatography (SEC HPLC, Supplementary Figure S5). Compared to other approaches to radiolabeling nanoparticles, this strategy is more facile and requires fewer steps.

#### **In Vitro and in Vivo Stability of MS2–Ab Conjugates.**

We tested 4 possible chelators (NOTA, NOTA-GA, DOTA, and DOTA-GA) to compare the stability of the complexes they form with the <sup>64</sup>Cu radioisotope ions. Similar stability was observed for all the chelators investigated, with NOTA showing a larger amount of signal associated with the intact capsid peak at longer time points. Based on these results, we decided to proceed with the NOTA–MS2 conjugates for *in vivo* experiments.

Radiolabeled conjugates were analyzed for purity and assembly state using SEC HPLC. The radioactivity traces indicated that most of the radioactive <sup>64</sup>Cu isotope remained associated with the intact capsids even upon incubation in mouse serum at physiological temperature (Figure 3, Supplementary Figure S5) or after circulating *in vivo* for 24 h (Supplementary Figure S6). Similar results were observed upon incubation in PBS alone (data not shown). MS2–antibody conjugates showed excellent stability at 37 °C (Supplementary Figure S7). This high level of intact capsid is encouraging for drug delivery applications, for which it is important to allow enough time for specific accumulation in the region of interest before releasing the active molecule. By housing the cargo on



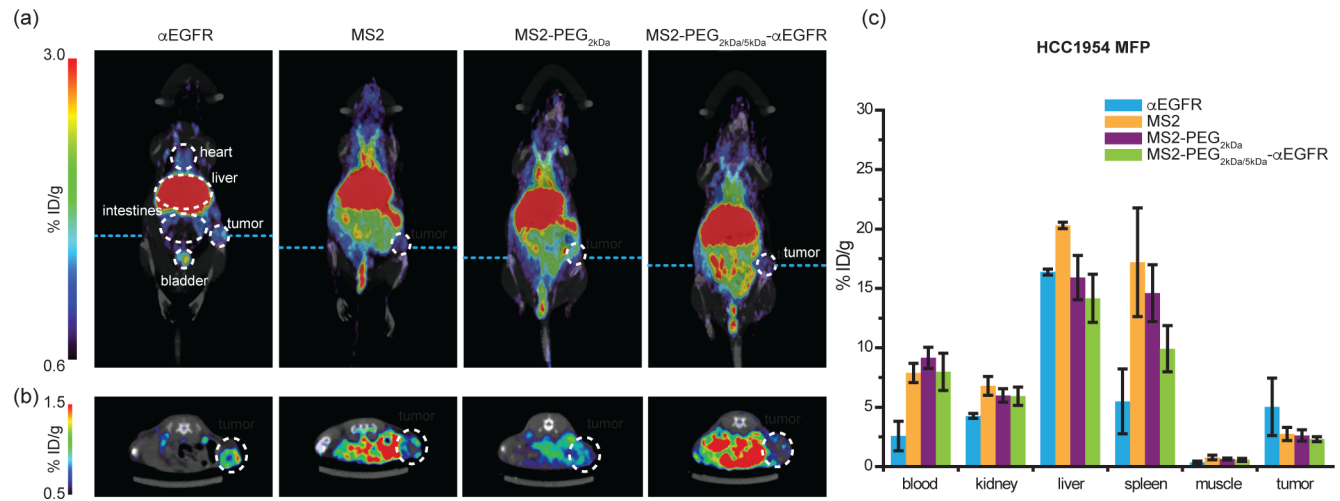
**Figure 3.** (a) Synthetic scheme for radiolabeling MS2 bacteriophage using the internal Cys residues (cutaway view). (b) Stability of MS2–NOTA-<sup>64</sup>Cu and radiolabeled MS2–antibody conjugate in mouse serum. The normalized signal from the radioactivity channel during SEC HPLC runs of aliquots taken at time points from 0 to 48 h indicates that the predominant species is the <sup>64</sup>Cu-labeled intact MS2 capsid. The secondary peak (\*) is likely due to partial disassembly or loss of <sup>64</sup>Cu and its interaction with other serum proteins.

the inside of the capsid, the side effects of systemic administration of chemotherapeutics could be mitigated. The second peak (shoulder at retention time ~6.3 min, Supplementary Figures S5, S6, and S7) can be attributed to the dissociation of <sup>64</sup>Cu from the chelator and nonspecific interactions with other serum proteins or to the partial disassembly of the MS2 capsids. Importantly, to allow for the capsids to be cleared from circulation, a gradual degradation is desired.

**Positron Emission Tomography.** Several molecular imaging approaches have been used for tracking the distribution of agents with therapeutic potential, including positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, bioluminescence, and fluorescence imaging.<sup>62</sup> Radionuclide-based imaging methods, especially PET, have been a particular focus in biomedical research due to advantages such as high sensitivity (picomolar level) and excellent tissue penetration by the signal.<sup>63</sup> Currently, most PET radiopharmaceuticals are based on small molecules, but their rapid clearance and poor specificity to tissues of interest are common drawbacks. In contrast, nanoparticles can have longer residence time in the body and possess sites that can be used for derivatization with radionuclides and targeting moieties. In addition, they have the potential to attain high specific activity (amount of radioactivity per mass of carrier), which is extremely important in order to achieve high-quality images at low doses of radioactivity. For our system, the specific activity was on average 1.1 GBq/mg capsid, as compared to 0.37–0.74 GBq/mg Fe reported for <sup>64</sup>Cu-DOTA–iron oxide nanoparticles,<sup>63</sup> and 15 GBq/mg reported for polymer nanoparticles.<sup>63</sup>

We used PET as a noninvasive way to gain information about the biodistribution of the MS2 conjugates in real time and at different time points while limiting the number of animals used. Dynamic imaging was performed from the time of injection until 1 h postinjection, and a 20 min static scan was collected at 24 h postinjection (Figure 4a,b).

The dynamic PET images taken from time of injection to 1 h postinjection were used to determine the clearance profile of



**Figure 4.** *In vivo* distribution of  $^{64}\text{Cu}$ -labeled MS2 scaffolds in nude mice with HCC1954 orthotopic breast cancer tumors (mammary fat pad, MFP). Mice were injected with 150–250  $\mu\text{Ci}$  radiolabeled agents.  $^{64}\text{Cu}$ -labeled antibodies were used as positive controls. (a) PET scans were acquired at 24 h postinjection. A maximum intensity projection (MIP) of the PET signal within a 50 mm slice is presented overlaid with a CT slice of 1 mm from the coronal plane. The horizontal blue dashed line represents the slice used for the transverse plane image in panel b. (b) A transverse plane through the tumor is shown to illustrate the agent distribution within the tumor. (c) Biodistribution studies were performed 24 h postinjection by harvesting the major organs, measuring their radioactivity using a scintillation counter and normalizing the radioactivity by the mass of the tissue. The error bars represent the standard deviation from triplicate samples.

the injected agents. A 3D region of interest (ROI) was chosen to overlap with the left ventricle of the heart to estimate the radioactivity present in the blood pool.<sup>64</sup> The signal showed a decay profile corresponding to a two compartment model, as evidenced by the shape of the  $\log_{10}$  plot of the signal within the ROI against the midpoint of each scanning frame (Supplementary Figure S9). The breathing motion and the heartbeat of the mouse, as well as the limited resolution of the animal scale PET scanner, made it difficult to determine the precise half-lives of the agents. However, the small slope of the curve at later time points (elimination phase) indicates a slow clearance for all agents. Surprisingly, no significant increase in circulation half-life was attained after PEG or antibody modification. The antibody had a shorter half-life than expected, which could be due to multiple modifications with the chelator leading to an altered clearance, or to dissociation of the  $^{64}\text{Cu}$  from the chelator.<sup>65,66</sup>

Throughout the experiment, a significant amount of signal was observed in the liver, the lower abdominal area, and the heart. Based on the signal in the heart and the biodistribution results, we concluded that the unmodified MS2 capsid and its derivatives remain in circulation for a prolonged time. At 24 h postinjection, the decay-corrected signal was significantly weaker overall, as result of the agents being cleared from the system (Figure 4a,b).

**Biodistribution Studies.** Mice were injected with each agent and sacrificed at either 1 or 24 h time points. The blood and major organs were harvested, and their activity was measured using a gamma counter. The tissue samples were weighed, and the radioactivity signal was normalized as percent injected dose per gram of tissue (% ID/g, Figure 4c and Supplementary Figure S9). To investigate the nonspecific tissue uptake of the agents, we calculated the tumor-to-muscle ratios by dividing the % ID/g in the tumor by the signal from the muscle tissue (Table 1). The numbers greater than 1 indicate specific uptake of the agents into the tumor. The free anti-EGFR antibody used as positive control had the highest tumor-to-muscle ratio. Notably, the amount of antibody injected was

**Table 1. Tumor-to-Muscle Ratio (T/M) of the Normalized Radioactive Signal per Unit Weight (% ID/g) Calculated as a Measure of Nonspecific Accumulation of the Agents<sup>a</sup>**

agent	T/M	
	MFP	SC
$\alpha$ EGFR	14.25 $\pm$ 5.71	23.83 $\pm$ 12.12
MS2	3.70 $\pm$ 1.04	2.55 $\pm$ 1.63
MS2-PEG <sub>2kDa</sub>	4.18 $\pm$ 1.09	3.52 $\pm$ 1.79
MS2-PEG <sub>2kDa/5kDa</sub> - $\alpha$ EGFR	4.62 $\pm$ 1.26	4.91 $\pm$ 2.72

<sup>a</sup>T/M = 1 indicates no specificity for the tumor over the muscle.

3–4 times more than the equivalent amount of antibodies attached to the MS2 capsids. In parallel studies (Supplementary Figure S10), we observed higher uptake of the free antibody control modified using the same protocol. However, the uptake of MS2 agents was very similar. The MS2 agents had tumor-to-muscle ratios in the range of 2.5–4.9, indicating specific uptake in the tumor compared to muscle tissue (Table 1). Overall tumor uptake for the MS2-based agents varied between 2 and 5% ID/g at 24 h postinjection (Figure 4c). The tissues with the largest degree of accumulation were the liver, the spleen (the usual clearance pathways for nanoparticles<sup>67</sup>), and the large intestine. The high signals in the blood even after 24 h of circulation validate the long half-lives observed from the analysis of the dynamic PET scans.

At 24 h postinjection, a similar amount of activity is present in the tumor for both the targeted and the untargeted viral capsids, suggesting that the extravasation from the blood vessel to the tumor might be the limiting step. Some recent studies have shown that a spherical structure is not ideal for crossing the gaps in the endothelial cell layer of tumor blood vessels.<sup>31,68</sup> Our results also indicate that targeting does not provide a significant improvement in the efficiency of the agent to accumulate in the region of interest, which has also been noted in the case of gold nanoparticles of similar size.<sup>17,69,70</sup> Similar results were observed in the case of liposomes,<sup>71</sup> although further studies investigating the delivery of drug molecules



indicated a clear benefit to the attachment of the targeting moiety,<sup>72</sup> presumably due to increased uptake once the cancer cells were reached.

The work presented herein is one of the few examples of antibody-targeted protein-based nanoparticles to be investigated for tumor homing. The particles exhibit surprisingly long circulation profiles and accumulate within the tumor environment. However, no significant difference is observed between the targeted and untargeted viral capsids, indicating that the diffusion through the endothelial gaps might be the limiting factor in determining tumor accumulation of nanoparticles. Our observations add evidence to the case that other factors, such as size, shape, and overall charge, might play a bigger role in the homing of nanoparticles to the tumor, while the targeting groups can increase specificity to tumor cells within the tumor environment. This hypothesis can help guide the design of nanoparticle carriers with various physical properties, while the synthetic plan and battery of analyses performed in this work can provide a framework for the thorough characterization of new scaffolds for drug delivery.

## CONCLUSIONS

*In vitro* results indicate specific binding of the MS2–Ab targeted agents to the EGFR receptor on the surface of cancer cell models. The agents can be radiolabeled to obtain high-specific-activity constructs that maintain structural integrity over 48 h when incubated in mouse serum at 37 °C. Upon intravenous injection in mouse breast cancer models, the MS2 viral capsid conjugates show moderate tumor uptake, comparable to targeted inorganic nanoparticles. No significant difference in tumor uptake is observed between the antibody-targeted and untargeted capsids.

Given the potential of the MS2 VLPs to carry up to 180 copies of drug molecule cargo, the large amount of agent accumulated in the tumor could provide a significant therapeutic effect. As such, the efficiency of MS2–antibody conjugates for delivering drug cargo and inhibiting tumor growth will be investigated. Recent studies have investigated the effects of size and shape of the carrier on the accumulation of the nanoparticle in the tumor.<sup>31</sup> Comparison studies with other virus-like particle shapes available (such as disks and rods) for their ability to deliver cargo to tumor environments are currently underway in our laboratory.

In this study, we have shown that MS2 is a robust and synthetically versatile platform that has a slow clearance profile *in vivo*. *In vitro* results suggest that the MS2 platform remains an attractive candidate for the synthesis of targeted agents for a variety of diseases. However, based on the limited tumor uptake *in vivo*, the targeting functionalities appended to these capsids might have to focus on disease targets that do not require extravasation, such as the treatment and monitoring of cardiovascular diseases and liquid tumors.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00566.

Synthetic schemes, agent characterization, flow cytometry experiments, live-cell microscopy, stability studies, and additional biodistribution experiments (PDF)

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

EGFR, epidermal growth factor receptor; PET, positron emission tomography; PEG, polyethylene glycol

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# Bioconjugation of Gold Nanoparticles through the Oxidative Coupling of *ortho*-Aminophenols and Anilines

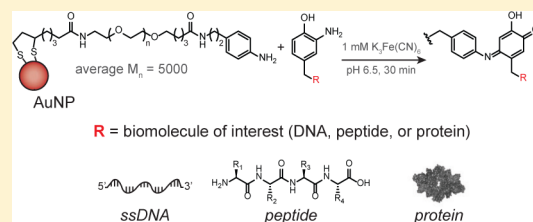
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## S Supporting Information

**ABSTRACT:** While there are a number of methods for attaching gold nanoparticles (AuNPs) to biomolecules, the existing strategies suffer from nonspecific AuNP adsorption, reagents that are unstable in aqueous solutions, and/or long reaction times. To improve upon existing AuNP bioconjugation strategies, we have adapted a recently reported potassium ferricyanide-mediated oxidative coupling reaction for the attachment of aniline-functionalized AuNPs to *o*-aminophenol-containing oligonucleotides, peptides, and proteins. The aniline-AuNPs are stable in aqueous solutions, show little-to-no nonspecific adsorption with biomolecules, and react rapidly (30 min) with *o*-aminophenols under mild conditions (pH 6.5, 1 mM oxidant).



## INTRODUCTION

The specific conjugation of AuNPs to biomolecules has applications in imaging,<sup>1,2</sup> drug delivery,<sup>3</sup> and enzyme immobilization.<sup>4–7</sup> As a result, a number of existing strategies have been developed for the covalent attachment of gold nanoparticles (AuNPs) to biomolecules.<sup>8–11</sup> Most commonly, these approaches take advantage of the strong thiol–AuNP interactions ( $\sim 200$  kJ/mol in bond energy),<sup>12</sup> allowing cysteine-containing peptides,<sup>13–15</sup> thiol-DNA,<sup>16</sup> and proteins with solvent accessible cysteines<sup>17,18</sup> to be attached to AuNPs. Proteins have also been adsorbed to negatively charged AuNPs through electrostatic interactions.<sup>19,20</sup> Affinity-based, non-covalent interactions, such as the biotin–streptavidin interaction,<sup>4,21</sup> and the His<sub>6</sub>/nickel–nitrilotriacetic acid (NTA) interaction,<sup>11,22,23</sup> have also been used to produce AuNP–protein conjugates. Furthermore, the covalent conjugation of proteins to suitably functionalized AuNPs has been demonstrated using EDC/NHS activation,<sup>5,24</sup> maleimide–thiol chemistry,<sup>25,26</sup> and Cu(I) catalyzed click chemistry.<sup>27</sup>

While these methods have been used successfully in many instances, they typically suffer from significant background AuNP adsorption, wherein positively charged biomolecules readily adsorb to the negatively charged particles. This can be problematic for chemisorption, electrostatic adsorption, affinity-based noncovalent interactions, and some covalent conjugation strategies in which the AuNP surface is not passivated. In addition, AuNPs functionalized with *N*-hydroxysuccinimide (NHS) esters and maleimides are susceptible to hydrolysis in aqueous solutions, and the reported Cu(I)-mediated click strategy<sup>27</sup> suffers from extended reaction times (3 days). As a result, we sought to improve the existing bioconjugation strategies by adapting a previously reported oxidative coupling between anilines and *o*-aminophenols.<sup>28,29</sup> The reaction proceeds using potassium ferricyanide as a protein-tolerant

oxidant in near neutral solutions (pH 6.5), and affords a single reaction product.<sup>29</sup> The involved precursors are stable in aqueous solutions, and the reaction proceeds quickly under mild conditions, allowing efficient coupling even at high dilution. As described herein, adapting this strategy for the bioconjugation of AuNPs has allowed us to overcome the limitations listed above to prepare functionalized particles for a wide variety of applications.

## RESULTS AND DISCUSSION

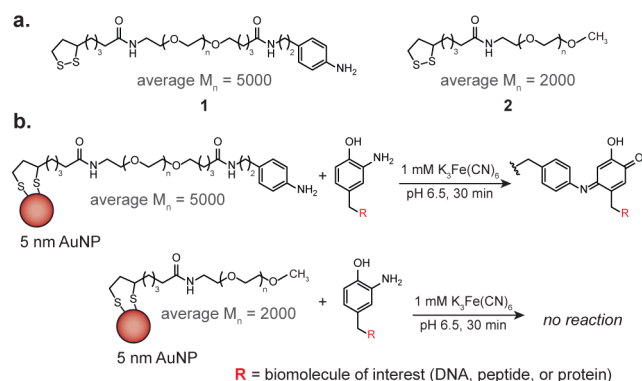
To produce aniline-coated AuNPs that are stable in aqueous solutions, a AuNP surface passivating ligand was designed. As previous reports have shown a decrease in nonspecific adsorption of biomolecules to polyethylene glycol (PEG) coated AuNPs,<sup>30</sup> the ligand was based on a core PEG5k chain. The PEG5k was capped on one end with a disulfide to enable attachment to the AuNP surface, and terminated on the opposite end with an aniline moiety to allow for subsequent reactivity with *o*-aminophenols.

The AuNP modification strategy was based on compound **1** in Figure 1a. A straightforward synthesis was developed to prepare the desired ligand. Briefly, commercially available valeric acid-PEG5k-amine was reacted with the NHS ester of lipoic acid to afford valeric acid-PEG5k-lipoamide. The valeric acid terminus was then functionalized with 2-(4-aminophenyl)-ethylamine in the presence of NHS and *N,N'*-dicyclohexylcarbodiimide (DCC) to produce the desired aniline-PEG5k-lipoamide ligand. As a negative reaction control, a methoxy-PEG2k-lipoamide ligand, **2**, was synthesized in an analogous manner.

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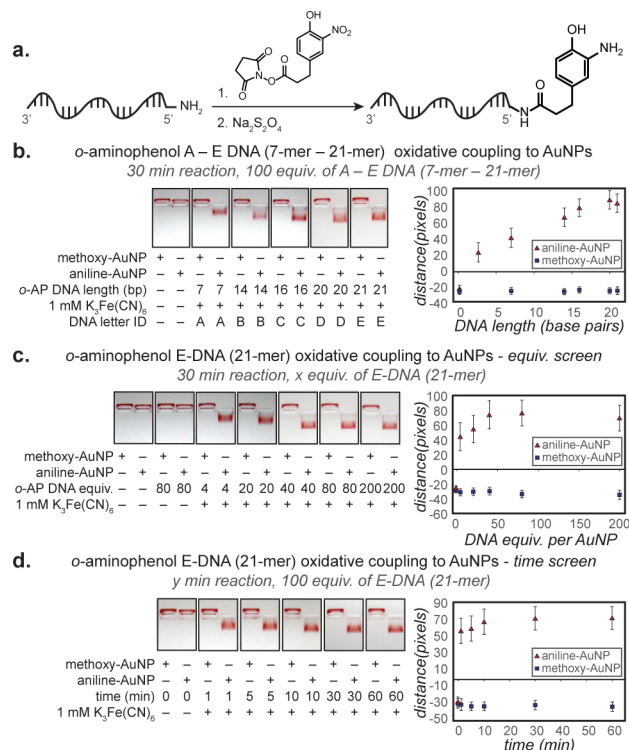


**Figure 1.** Strategy for the covalent attachment of biomolecules of interest to AuNPs (5 nm in diameter): (a) PEG-based nanoparticle passivating ligands were prepared with distal aniline (**1**) or methoxy (**2**) groups; (b) aniline-PEG5k-labeled AuNPs and *o*-aminophenol-containing DNA, peptides, or proteins were combined in the presence of potassium ferricyanide to afford stable conjugates. Methoxy-PEG2k-labeled AuNPs served as a control.

Overnight incubation of the aniline-PEG5k-lipoamide or methoxy-PEG2k-lipoamide with citrate-stabilized 5-nm-diameter AuNPs in a slightly basic solution (pH 8.5) afforded aniline- or methoxy-AuNPs, as shown in Figure 1b. The addition of the ligand was confirmed for both the aniline- and methoxy-coated AuNPs by an increase in the AuNP average diameter, as determined by dynamic light scattering (DLS, Supporting Information Figure S1) and a decrease in electrophoretic mobility of the AuNPs on a native agarose gel. The resulting particles were stable in solution for weeks, and resistant to precipitation at high salt concentrations (Supporting Information Figure S2).

As noted above, electrostatic attraction is often used to attach antibodies and other proteins to AuNPs. However, in other contexts these interactions render it difficult to attach biomolecules to specific sites. To evaluate nonspecific protein adsorption to the aniline-coated AuNPs and methoxy-coated AuNPs, which would impair our analysis of specific protein attachment, a selection of proteins (lysozyme, horseradish peroxidase, bacteriophage MS2, and bovine serum albumin) was incubated with bis(*p*-sulfonatophenyl)phosphine dehydrate dipotassium salt (BSPP) stabilized AuNPs, methoxy-coated AuNPs, and aniline-coated AuNPs overnight. Subsequent native agarose gel analysis allowed determination of nonspecific protein adsorption (Supporting Information Figure S3). Significant adsorption was observed for BSPP stabilized AuNPs with each of the proteins, but there was no observed protein adsorbed to the methoxy-AuNPs or aniline-AuNPs. With evidence that background adsorption would not be a major concern for the aniline-AuNPs or the control methoxy-AuNPs, the ability of these aniline-coated AuNPs to undergo oxidative coupling with *o*-aminophenol containing DNA, peptides, and proteins was evaluated.

Amine (5') oligonucleotides were labeled with an *o*-nitrophenol NHS ester, followed by reduction with  $Na_2S_2O_4$  to afford *o*-aminophenol DNA (Figure 2a). The sequences consisted of 7 nt (A), 14 nt (B), 16 nt (C), 20 nt (D, E, and F'), and 21 nt (E). The identity of these species was confirmed by MALDI-TOF MS (sequences A–F, Supporting Information Figure S4, S5). Reactions between *o*-aminophenol labeled oligonucleotides and aniline-AuNPs or the control methoxy-AuNPs were monitored by native agarose gel, as shown in



**Figure 2.** DNA attachment to aniline-AuNPs (5 nm in diameter). (a) Strategy for the introduction of an *o*-aminophenol to DNA sequences of interest. Amine (5') oligonucleotides were labeled with an *o*-nitrophenol *N*-hydroxysuccinimide ester, followed by reduction with  $Na_2S_2O_4$  to afford 5'-*o*-aminophenol DNA, as confirmed by MALDI-TOF MS (Supporting Information Figure S4). The electrophoretic mobility of the DNA-AuNP constructs was monitored by native agarose gel electrophoresis. The distance traveled toward the positive electrode (recorded as number of pixels from origin, 0 min = no addition of oxidant) was plotted for (b) varying lengths of DNA (7–21 bp), (c) DNA equivalents per AuNP (4–200 equiv), and (d) reaction time (0 to 60 min). Attachment of longer oligonucleotides resulted in a greater electrophoretic mobility of the DNA-AuNP conjugates. The maximum electrophoretic mobility of the conjugates was achieved at ~40 equiv of DNA per AuNP and a 30 min reaction time. The vertical bars represent the width of each band, thus specifying a range of values.

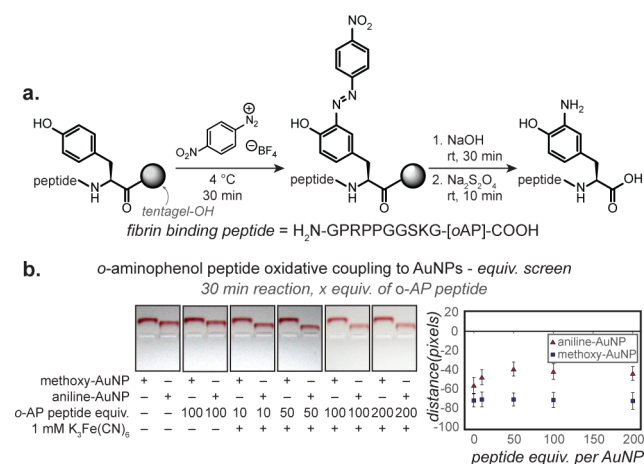
Figure 2b–d. A gel shift was observed only for aniline-AuNPs in the presence of oxidant and an *o*-aminophenol labeled oligonucleotide. The distance traveled toward the positive electrode (recorded as the number of pixels from the origin in a digital photograph of the gel) was plotted for varying lengths of DNA (7–21 bp, Figure 2b), DNA equivalents per AuNP (4–200 equiv, Figure 2c), and reaction times (0 to 60 min, Figure 2d). The attachment of longer oligonucleotides resulted in a greater electrophoretic mobility of the DNA-AuNP conjugates, presumably due to an increase in negative charge on the AuNPs. The maximum electrophoretic mobility of the conjugates was achieved at ~40 equiv of DNA per AuNP and a 30 min reaction time. Additionally, an increase in average particle diameter was observed for aniline-AuNPs coupled to *o*-aminophenol oligonucleotides in the presence of oxidant, as determined by DLS (Supporting Information Figure S1). The increase in average diameter indicated a high density of DNA strands on the AuNP surface. A similar size increase was not observed for the corresponding controls that were coated with



methoxy-terminated PEG chains (Supporting Information Figure S1).

The ability of DNA labeled AuNPs to hybridize with complementary oligonucleotides was also evaluated. AuNPs were labeled with 20 nt sequence F, followed by incubation with its complement, F' DNA. A slight increase in electrophoretic mobility was observed, as determined by native agarose gel (Supporting Information Figure S6). Analogous results were obtained when F'-coated AuNPs were incubated with sequence F. An increase in electrophoretic mobility was not observed upon incubating F-AuNPs or F'-AuNPs with noncomplementary oligonucleotides (Supporting Information Figure S6). Furthermore, incubation of F-AuNPs and F'-AuNPs in equal concentrations resulted in an increase in the overall average particle size in solution (as determined by DLS, Supporting Information Figure S1), indicating that the AuNPs labeled with complementary DNA are able to hybridize with each other to form large clusters in solution.

An *o*-aminophenol containing peptide was next tested for its attachment to aniline-AuNPs, as shown in Figure 3. A fibrin-



**Figure 3.** Peptide attachment to aniline-AuNPs (5 nm diameter). (a) Strategy for the introduction of an *o*-aminophenol on the fibrin-binding peptide,  $\text{H}_2\text{N-GPRPPGGSKG-COOH}$ . The peptide was synthesized using standard solid-phase peptide synthesis chemistry. A C-terminal tyrosine residue was modified with 4-nitrobenzenediazonium tetrafluoroborate to afford the azo peptide, which was cleaved from the resin with base and reduced to the *o*-aminophenol with  $\text{Na}_2\text{S}_2\text{O}_4$ . Masses were confirmed by MALDI-TOF MS (Supporting Information Figure S7). (b) The electrophoretic mobility of the peptide-AuNP construct was monitored by native agarose gel electrophoresis. Distance traveled toward the positive electrode (recorded as number of pixels from origin) was plotted for increasing peptide equivalents per AuNP (10–200 equiv). The maximum electrophoretic mobility of the conjugates was achieved at  $\sim 50$  equiv peptide. All peptide-AuNP reactions proceeded at rt for 30 min. The vertical bars represent the width of each band, thus specifying the range of values.

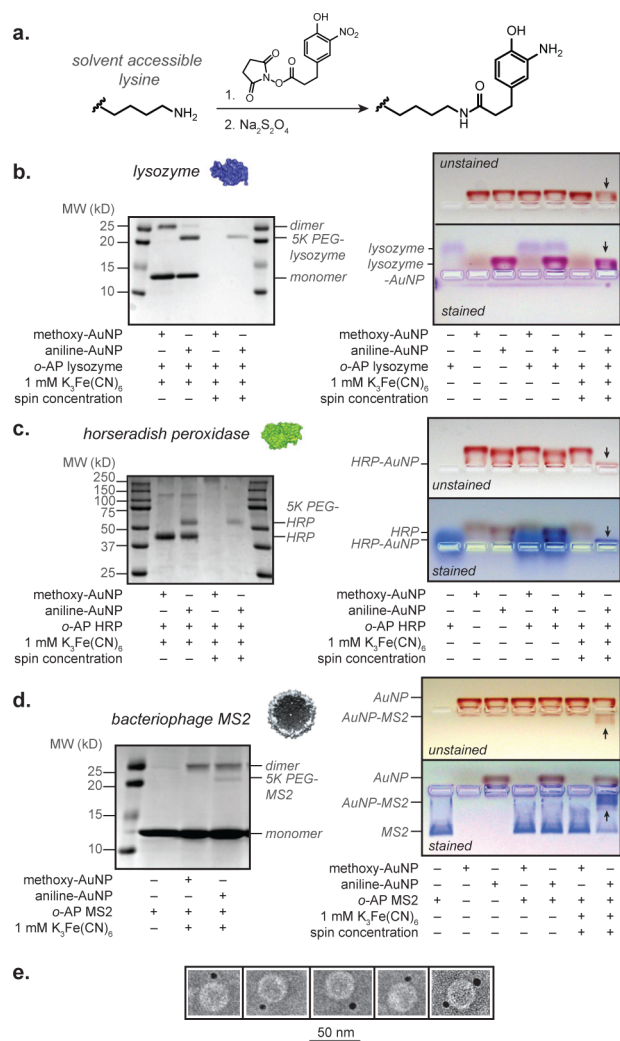
binding peptide,  $\text{H}_2\text{N-GPRPPGGSKG-COOH}$ ,<sup>31,32</sup> was synthesized using standard solid-phase peptide synthesis chemistry on Tentagel-OH resin. Following side chain deprotection with TFA, the C-terminal tyrosine residue was modified with 4-nitrobenzenediazonium tetrafluoroborate to afford an azo peptide that was cleaved from the resin with base and reduced to the *o*-aminophenol with  $\text{Na}_2\text{S}_2\text{O}_4$  (Figure 3a).<sup>32</sup> The molecular weights of these synthetic intermediates were confirmed by MALDI-TOF MS (Supporting Information

Figure S7). The *o*-aminophenol terminated peptide was then coupled to aniline-AuNPs. The electrophoretic mobility of the peptide-AuNP construct was monitored by native agarose gel (Figure 3b). A change in mobility was observed only for the *o*-aminophenol peptide in the presence of aniline AuNPs and oxidant. The distance traveled toward the positive electrode (recorded as the number of pixels from the origin in a digital photograph of the gel) was plotted for increasing peptide equivalents per AuNP (10–200 equiv). All peptide coupling reactions proceeded at rt for 30 min. The maximum electrophoretic mobility of the conjugates was achieved at  $\sim 50$  equiv of peptide. An increase in average particle diameter was observed for the peptide-AuNP conjugates, as determined by DLS (Supporting Information Figure S1). The size increase indicated a high density of peptide coverage on the AuNP surface.

Next, *o*-aminophenol labeled proteins were tested for their attachment to aniline-AuNPs, as shown in Figure 4. Lysozyme, horseradish peroxidase, and bacteriophage MS2 were labeled with an *o*-nitrophenol NHS ester, as confirmed by ESI MS. For each protein, 1–3 modifications were observed (Supporting Information Figure S8). The *o*-nitrophenol groups were then reduced with  $\text{Na}_2\text{S}_2\text{O}_4$  to afford *o*-aminophenol labeled lysozyme, horseradish peroxidase, and bacteriophage MS2 (Figure 4a).

The coupling reactions between *o*-aminophenol proteins and aniline- or methoxy-AuNPs were first monitored by native agarose gel electrophoresis. A minimal change in electrophoretic mobility was observed for the coupling of aniline AuNPs to *o*-aminophenol lysozyme (Figure 4b), while a clear change in electrophoretic mobility was visualized for both horseradish peroxidase and bacteriophage MS2 (Figure 4c,d). We attribute these differences to the number of equivalents used per AuNP in each reaction. Three, twenty, and ten equivalents of protein relative to AuNPs were used for the lysozyme, horseradish peroxidase, and bacteriophage MS2 coupling reactions, respectively. In the case of lysozyme, coupling reactions using more than 3 equiv of protein relative to AuNP resulted in conjugates that precipitated from solution. We attribute this instability to a significant change in surface charge and potential cross-linking between the particles. This possibility could be prevented using proteins bearing single *o*-aminophenol groups. Similar issues of conjugate instability were not observed in the case of horseradish peroxidase and bacteriophage MS2, allowing for greater equivalents to be used per reaction. The modest mobility shifts for lysozyme likely resulted from the small number of proteins on each particle.

As an additional characterization, the protein-AuNP conjugates were boiled in a solution of DTT and SDS (loading buffer) to reduce the AuNP-thiol bonds, affording the protein-polymer conjugate and precipitated gold aggregates (Figure 4b–d). This solution was then applied directly to an SDS-PAGE gel, revealing protein-polymer conjugates only when aniline-AuNPs were used. Consistent with the mobility experiments above, no protein-polymer conjugates were observed for the methoxy-AuNPs. In addition, the crude reaction mixture for lysozyme-AuNP and horseradish peroxidase-AuNP was subjected to 5–10 rounds of centrifugal filtration against 100 kDa MWCO filters to retain AuNP-protein conjugates and remove any unbound, unmodified protein. The removal of the unmodified protein was confirmed by SDS PAGE. To ensure that the protein-polymer conjugates



**Figure 4.** Protein attachment to aniline-AuNPs. (a) Strategy for the introduction of an *o*-aminophenol to proteins that contain solvent accessible lysine residues, including (b) lysozyme, (c) horseradish peroxidase, and (d) bacteriophage MS2. AuNP-protein conjugates had a slightly lower electrophoretic mobility than the corresponding controls when visualized by native agarose gel electrophoresis (right column, conjugate indicated by black arrow). PEGSk-protein conjugates were visualized through SDS PAGE gel electrophoresis (left column). Excess unmodified lysozyme and horseradish peroxidase were removed through successive centrifugal filtrations in 100 kDa MWCO filters. (e) MS2-AuNP conjugates were further characterized using TEM (scale bar = 50 nm).

were not simply retained in the 100 kDa MWCO filters due to their increase in hydrodynamic volume, lysozyme-PEGSk and horseradish peroxidase-PEGSk conjugates were independently synthesized and subjected to successive rounds of centrifugal filtration with 100 kDa MWCO filters. The protein-polymer conjugates were not retained in the filters (Supporting Information Figure S9), providing further support that the protein-polymer-AuNP conjugates were retained following successive rounds of centrifugal filtration only due to their attachment to the AuNPs. A similar strategy for removing unbound protein was not possible for bacteriophage MS2-AuNP conjugates because unmodified MS2 is retained by 100 kDa MWCO filters. Instead, the AuNP-MS2 conjugate was extracted from the native agarose gel and further characterized

by transmission electron microscopy, revealing 1–2 AuNPs per capsid (Figure 4e; Supporting Information Figure S10).

## CONCLUSIONS

This strategy presents a promising new method for the attachment of aniline-AuNPs to *o*-aminophenol-labeled biomolecules of interest. The reaction proceeds under mild conditions (pH 6.5, 1 mM oxidant) with fast reaction times (30 min) and little-to-no background protein adsorption to the AuNPs. In future experiments, we hope to use this strategy for the production of AuNP-biomolecule conjugates for imaging applications and for the immobilization of enzymes. We also plan to extend this method to include other thiophilic inorganic nanomaterials such as silver nanoparticles, CdSe/ZnS quantum dots, CdSe/CdS dot-rods, and certain Au-coated magnetic nanoparticles,<sup>33</sup> for the production of stable inorganic nanomaterial bioconjugates.

## ASSOCIATED CONTENT

### Supporting Information

Full experimental details, additional gel analysis, dynamic light scattering traces, transmission electron microscopy images, <sup>1</sup>H NMR, and mass spectrometry data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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