Nanosensing protein allostery using a bivalent mouse double minute two (MDM2) assay

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The tumor suppressor protein, p53, is either mutated or absent in >50% of cancers and is negatively regulated by the mouse double minute (MDM2) protein. Understanding and inhibition of the MDM2-p53 interaction are, therefore, critical for developing novel chemotherapeutics, which are currently limited because of a lack of appropriate study tools. We present a nanosensing approach to investigate full-length MDM2 interactions with p53, thus providing an allosteric assay for identifying binding ligands. Surface-enhanced Raman scattering (SERS)-active nanoparticles, functionalized with a p53 peptide mimic (peptide 12.1), display biologically specific aggregation following addition of MDM2. Nanoparticle assembly is competitively inhibited by the N-terminal MDM2-binding ligands peptide 12.1 and Nutlin-3. This study reports nanoparticle assembly through specific protein–peptide interactions that can be followed by SERS. We demonstrate solution-based MDM2 allosteric interaction studies that use the full-length protein.

Raman spectroscopy | biosensing | nano-assembly | protein interaction studies | assay system

The p53 tumor suppressor protein is often referred to as the “guardian of the genome” owing to its key role in cell-cycle regulation and its activity in a number of different cancer pathways (1–3). The antiproliferative action of p53 arises from the induction of cell-cycle arrest and apoptosis in cells subjected to DNA damage in response to stress. As such, p53 is central to protecting cells from uncontrolled growth and malignant transformation with inactivation or mutation of p53 found in over 50% of human cancers (1–3). Under nonstressed conditions, mouse double minute (MDM2) negatively regulates p53, primarily through the ubiquitin degradation pathway but also via transrepression (4, 5). Studies have shown up-regulation of MDM2 in malignancies where p53 is fully functional, making the MDM2-p53 feedback loop of great interest in chemotherapeutic studies (6, 7).

MDM2 is a multidomain protein that exerts E3-ligase activity on a number of proteins, including p53 (5, 8, 9). The ubiquitination activity of MDM2 is a multisubunit process whereby the N-terminal hydrophobic pocket and central acidic domain of MDM2 are used in p53 binding, to the N-terminal and central domains of p53, respectively (10). The complexity of this multisubunit interaction makes it relatively difficult to investigate the allosteric nature of MDM2. This is made more difficult by the problems in acquiring a structure of full-length MDM2 because of intrinsically disordered regions on the protein that reduce likelihood of crystallization. Focus solely on the N-terminal domain of MDM2 has identified Nutlin-3-type molecules that activate p53 in cells; however, these also act as allosteric agonists that stimulate rather than inhibit p53 ubiquitination (10–15).

The putative dimerization of MDM2 through the C-terminal really interesting new gene (RING) domain is reported to be critical for E3-ligase activity; however, molecular reasoning for this is not fully understood (8, 16–18). To date, studies on MDM2 structure have been conducted with purified domain constructs, which is not representative of full-length protein activity in nature.

Using full-length MDM2, which has the capacity to exhibit allosteric interactions (19, 20), would provide an assay for the screening of MDM2 ligands. Domain construct studies have investigated the multiple binding interactions of MDM2, but few, thus far, have used unlabeled full-length protein to simultaneously interrogate two binding events. The assay described in this report monitors both N-terminal and C-terminal activities of MDM2 simultaneously using full-length protein, which is critical to understanding the biological action of the native protein. The methodology provides a step forward in the capability for investigating such intricate interactions, and a unique insight is provided into the allosteric nature of native MDM2 that cannot be observed using other techniques.

Current methodologies used to probe interactions in biological systems commonly use readout tools such as fluorescence (21–23). This technique is subject to a high background from biological media and is limited by broad emission bands and the inability to probe interactions over distances of more than 10 nm. Surface-enhanced Raman scattering (SERS) is a vibrational spectroscopy that can provide similar sensitivity to fluorescence, but narrower spectral peaks allow for simultaneous detection of multiple species (24, 25). SERS interrogates an analyte, adsorbed onto a roughened metal surface, from which further Raman signal enhancement is achieved by coinciding the laser excitation wavelength with the analyte absorbance maxima (26, 27). SERS can also be enhanced or “turned on” by aggregation of nanoparticles (NPs) in solution because of “hot spots” of higher electromagnetic fields at NP junctions (26–28). Cotton et al. pioneered the use of NP and SERS for proteomic investigations and developed the first SERS-based enzyme immunoassay in 1989 (29). Subsequently, a number of SERS-based immunoassays have been documented (30–39). Assembly of functionalized NPs via protein–ligand interactions has been previously demonstrated using techniques such as excitation spectroscopy, transmission electron microscopy, and gel electrophoresis, all of which lack the sensitivity of SERS (40–44). Such interactions have been detected via SERS on metal surfaces, where binding events are compromised by protein orientation and conformation at the surface. Solution-based studies allow structural integrity of the full-length protein to be maintained, thus providing accurate information about complex interactions; however, such investigations have yet to be reported (36).

In this report, we outline a method for NP assembly through the interaction of full-length MDM2 with an N-terminal-domain peptide ligand. By exploiting the sensitivity and selectivity of SERS, we provide insight into the allosteric nature of MDM2 in cell-cycle regulation and its activity in a number of different cancer pathways (1–3). The antiproliferative action of p53 arises from the induction of cell-cycle arrest and apoptosis in cells subjected to DNA damage in response to stress. As such, p53 is central to protecting cells from uncontrolled growth and malignant transformation with inactivation or mutation of p53 found in over 50% of human cancers (1–3). Under nonstressed conditions, mouse double minute (MDM2) negatively regulates p53, primarily through the ubiquitin degradation pathway but also via transrepression (4, 5). Studies have shown up-regulation of MDM2 in malignancies where p53 is fully functional, making the MDM2-p53 feedback loop of great interest in chemotherapeutic studies (6, 7).

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solution, which is not achievable by current methodologies, which only monitor one binding interaction, involve protein labeling, or require surface immobilization.

Results

Protein–Peptide Nanoparticle Assembly. We propose a solution-based approach to investigate the interaction of full-length MDM2 with a p53 peptide mimic exploiting the sensitivity and selectivity of SERS, p53 is known to bind the N-terminal hydrophobic pocket of MDM2 by forming an amphipathic helix containing the peptide motif, FxxWxxL (45). Peptide 12.1 (MPRFMDYWEGLN) originates from a library of p53 peptide mimics and demonstrates high-affinity binding to the MDM2 hydrophobic cleft (46). Tryptophan, a key residue involved in aromatic-aromatic interactions with MDM2 (47, 48), was replaced with alanine to create the mutant peptide 12.1WΔA, which provided a negative control to ensure biological specificity of the interaction. In theory, putative dimerization of full-length MDM2 (through its RING domain) would present two N-terminal hydrophobic pockets free (per dimer) to interact with ligands such as peptide 12.1. N-terminal interactions with peptide ligand, therefore, allow the MDM2 dimer to bring together two peptide 12.1-functionalized silver NP, peptide silver nanoparticle (PSN)-12.1, in solution (Fig. 1A, i). An alternative model for PSN-12.1 assembly, via a secondary peptide 12.1-binding site, is also illustrated (Fig. 1A, ii). The latter is unlikely because there is no evidence for two different peptide 12.1-binding sites on MDM2, and cell-based studies suggest MDM2 is oligomeric in solution (16). MDM2-induced aggregation can be monitored over time by extinction spectroscopy and premodifying peptide 12.1 with a benzotriazole Raman tag, BT, enables the

![Fig. 1.](image-url) (A) Schematic of the proposed assembly of PSN through specific interactions between MDM2 and peptide 12.1 (not to scale). Inset shows two proposed models for MDM2 bivalency resulting in PSN aggregation from a MDM2 dimerization (e.g., “oligomeric”) conformation (i) and monomeric MDM2 (ii) have two distinct binding sites with the known and an alternative peptide 12.1 binding site (not to scale). (B and C) Extinction spectroscopy (B) and SERS analysis (C) before (dashed) and after (solid line) addition of MDM2 to PSN-12.1.
process to be explored using SERS. Both extinction and SERS intensity can be monitored over time to investigate the biological interactions driving this NP-assembly process.

**Peptide Silver Nanoparticles.** Reproducibility of spectra from Raman reporters used in SERS-based immunoassays is extremely important to obtain reliable results. Benzotriazole dyes adsorb onto silver nanoparticle surfaces in the same orientation irrespective of concentration, resulting in reproducible Raman spectral intensities (49). This is attributable to steric hindrance presented by covalent interactions between N1 and N3 lone electron pairs with the silver surface (50, 51). BT has an absorbance $\lambda_{\text{max}}$ of 487 nm and, as such, is close in resonance when using an excitation wavelength of 514 nm. The most prominent peak in the BT Raman spectrum occurs at a shift of 1,416 cm$^{-1}$ (Fig. 1C) and can be attributed to the azo stretch in the dye structure (Fig. S1).

Peptide 12.1 and mutant peptide 12.1$\Delta A$ were modified with BT and directly conjugated to EDTA-reduced silver NP (AgEDTA) in a one-step reaction. BT consists of a triazole moiety with an affinity for silver surfaces, a Raman active chromophore, and a stabilizing polyethylene glycol spacer (Fig. S1). Circular dichroism (CD) analysis of peptide 12.1 and BT-modified peptide 12.1 showed the modification to have no inhibitory effect on the peptide adopting a helical conformation (Fig. S2). BT-modified peptide 12.1 and mutant peptide 12.1$\Delta A$ displayed high- and low-level binding, respectively, to MDM2 in ELISA competition assays (Fig. S3). Successful PSN formation was shown by an increase in particle size through extinction spectroscopy, and matrix-associated laser desorption ionization-mass spectroscopy (MALDI-MS) analysis was used to identify BT-peptide molecules anchored on the nanoparticle surface (Figs. S4 and S5).

**MDM2-induced PSN Aggregation.** Unlabeled full-length MDM2 was added to PSN solutions at various concentrations based on a molar excess of protein to PSN. This is in line with previously published data using streptavidin for controlled assembly of biotin-functionalized gold NP (40). Aggregation of 15 pM PSN-12.1 in solution was observed following the addition of MDM2 concentrations up to a NP: protein ratio of 1:1,000 (15 pM PSN, 15 nM MDM2) (Figs. 2 and 4 and SI Text). This confirms the importance of specific amino acid residues in the binding event between MDM2 and NP-bound peptide. At protein concentrations higher than this, the extent of aggregation between PSN-12.1 and PSN-12.1$\Delta A$ solutions cannot be distinguished by extinction spectroscopy or dynamic light scattering (Figs. 2 and 3). At these concentrations, MDM2 is present in such excess that binding to the mutant peptide 12.1$\Delta A$ is comparable to that of peptide 12.1, indicating a saturation point to the assay. Extinction spectroscopic measurements of samples taken 96 h after MDM2 addition show a much greater distinction in aggregation extent between PSN-12.1 and the mutant PSN-12.1$\Delta A$ up to the saturation concentration of 15 nM MDM2 (Fig. 24); however, such a time duration for an immunoassay is impractical. The PSN assembly process can be monitored over time by measuring the extinction change at the $\lambda_{\text{max}}$, and aggregation was seen to plateau within the first 30 min (Fig. 34). These data correlate with previous literature describing binding of p53 BOX-I to the MDM2 hydrophobic cleft to be a stable, high-affinity interaction. PSN-12.1 assembly can be interpreted through either of the models proposed in Fig. 1; however, because there is no evidence to support the latter, the most likely explanation for the observed PSN aggregation is an active MDM2-dimer (or “oligomer”).

**MDM2-Induced Aggregation “Turns On” SERS.** Initial SERS studies involved analysis of PSN samples after completion of aggregate assembly monitored by extinction spectroscopy. SERS enhancement was measured by comparing the standardized peak height at 1,416 cm$^{-1}$ (Fig. 1B) for each sample in relation to unaggregated PSN solutions. Raman signal intensity increased in a positive correlation with MDM2 concentration up to a molar ratio of 1:1,000 PSN:MDM2 (15 pM PSN, 15 nM MDM2) (Fig. 4). This corresponds with previous extinction spectroscopy investigations indicating assay saturation at this concentration. When MDM2 was present at a larger molar excess than 1:1,000, a decrease in SERS intensity was observed (Fig. 4A). At these concentrations, protein can form almost monolayer coverage on the PSN surface, thus dampening Raman signals associated with BT but still allowing aggregation, as seen by extinction spectroscopy (Fig. 3B). Another possibility is that large aggregates

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**Fig. 2.** Extinction spectroscopy and particle size analysis of PSN solutions before and after addition of MDM2. (A and B) Extinction profiles (A) and particle size distributions (B) of PSN-12.1 (i) and PSN-12.1$\Delta A$ (ii) samples without MDM2 (black) and 96 h after addition of 300 (dotted gray), 500 (short dashed gray), 1,000 (long dashed gray), and 2,000 (solid gray) MDM2 monomers per PSN.
form that fall out of solution; however, this explanation is contradicted by the partial aggregation observed in scanning electron microscopy (SEM) (Fig. S6). At a molar ratio of 1:1,000 PSN: MDM2 (15 pM PSN, 15 nM MDM2), an eightfold increase in peak height was observed compared with samples where protein was absent. No such signal enhancement was apparent for PSN-12.1 (Fig. 4A) and SERS analysis of MDM2-induced PSN aggregation enables protein detection at a molar excess of 100 MDM2 per PSN (15 pM PSN, 1.5 nM MDM2), demonstrating a lower limit of detection than was achievable using extinction spectroscopy (Figs. 3B and 4A). Comparable results for PSN-12.1 and PSN-12.1WΔA samples were also more distinguishable when applying SERS rather than extinction spectroscopy (Figs. 3 and 4). These data indicate that SERS presents a more sensitive analysis technique than extinction spectroscopy for NP assembly controlled by biological interactions.

**Temporal SERS Analysis of Aggregation.** Our findings show that the assembly of PSN-12.1 with MDM2 is a time- and concentration-dependent process (Fig. 3). To this end, SERS analysis was investigated to monitor the PSN assembly process over time. To minimize SERS signal variations, PSN preparation and final solution concentrations were optimized. Focus of the laser through the bulk of the solution and continuous sample rotation ensured consistent sampling of the components throughout the duration of the experiment.

The rate at which SERS intensity reached saturation was seen to increase with MDM2 concentration, and it can be interpreted from the data that the formation of PSN assemblies approaches completion within 11, 9, and 7 min for PSN:MDM2 ratios of 1:300, 1:500, and 1:1,000, respectively (15 pM PSN, 4.5–15 nM MDM2) (Fig. 4B). This is quicker than observed in extinction spectroscopy (Fig. 3A), demonstrating the higher sensitivity of SERS as an analytical tool for monitoring NP aggregation. These data demonstrate that SERS can be used as a viable tool for monitoring time-dependent NP assembly, although it must be realized that it does not represent the system found in nature owing to PSN solution kinetics.

**Inhibition of the MDM2-PSN Interaction.** To test the potential for development of the assay for investigating MDM2 interactions with small molecules, we exposed MDM2 to N-terminal-binding ligands to competitively inhibit the aforementioned PSN-12.1 assembly. MDM2 is likely to exhibit a higher binding affinity to free peptide 12.1 (inhibitor 12.1) than to NP-bound peptide 12.1, because of the solution kinetics. Nutlin-3 is a potent and well studied small molecule inhibitor of the MDM2-p53 interaction (15). For all inhibition experiments, a molar ratio of 1:1,000 PSN: MDM2 (15 pM PSN, 15 nM MDM2) was used owing to the large extent of the aggregation observed (Figs. 2–4). MDM2 was preincubated with a 100-fold molar excess of inhibitor (0.96 mM MDM2, 96 nM inhibitor) before mixing with PSN-12.1 solutions (6.25 mM added to 400 mM of 15 pM PSN), and temporal analysis was carried out using extinction spectroscopy. A lesser decrease in the plasmon band was observed for PSN-12.1 solutions treated with MDM2 preincubated with either inhibitor 12.1 or Nutlin-3 than was evident following the addition of native MDM2 (Fig. 5). A much greater enhancement in Raman signal was also observed in the presence of native MDM2 compared with MDM2 preincubated with inhibitor (Fig. 5B). Inhibitor 12.1 and Nutlin-3 were able to bind the MDM2 hydrophobic cleft, thus blocking the binding site for peptide 12.1 molecules on PSN-12.1 and disallowing MDM2-mediated PSN assembly. Signal changes in SERS and extinction spectroscopy associated with PSN aggregation were, therefore, not observed to the same extent (Fig. 5B). It was subsequently observed that lowering the excess of inhibitor resulted in a decrease in inhibition efficiency by extinction spectroscopy and SERS (Fig. 5C). Varying the molar excess of inhibitor 12.1 prebound to MDM2 in this way demonstrates PSN-assembly inhibition in a dose-dependent manner. The extinction spectroscopy and SERS data, together, demonstrate a competitive inhibition of MDM2–peptide 12.1-driven NP assembly by inhibitor 12.1 and Nutlin-3. A 1.4x increase in SERS response was detected at the highest concentration of inhibitor 12.1, suggesting that some PSN assembly occurred (Fig. 5C). Depletion in extinction plasmon band for the same sample in extinction spectroscopy is negligible (Fig. 5C), thus identifying SERS as the superior technique to investigate inhibitor potency. SERS analysis also provides a positive response for NP assembly that is
Fig. 5. Competitive inhibition of MDM2-induced PSN aggregation. Schematic illustrates proposed MDM2-induced PSN aggregation inhibition with inhibitor peptide 12.1 and Nutlin-3. (A and B) X-fold decrease in the plasmon band monitored by extinction spectroscopy (light gray (A) and X (B)) and x-fold SERS intensity change following 30 min incubation of MDM2 with varying concentrations of inhibitor molecule [dark gray (A) and circle (B)].

Disruption of the Dimerization Interface. To further test the assay capabilities for investigating MDM2 and verify the requirement for MDM2 oligomerization in PSN assembly, MDM2 was preincubated with self-peptides from one linear motif that stabilizes the MDM2/MDMX heterodimer at the N-terminal junction of the C-terminal RING domain (amino acids 430-LPLNAI-435) (8) at a molar ratio of 1:100 MDM2:peptide (0.96 mM MDM2, 96 mM peptide). SERS analysis of PSN solutions (15 pM) was carried out following the addition of MDM2 preincubated with peptides 43 (DKEESVSSLPLNAI) and 44 (PLNAIEPCVICQGRP) using extinction spectroscopy (Fig. S9). Preincubation of MDM2 with peptides 43 and 44 resulted in a decrease in PSN-12.1 assembly. Despite both dimerization-motif peptides demonstrating an inhibitory effect on PSN-12.1 assembly, a 1.6-fold increase in SERS was observed in the presence of peptide 43 compared with a 1.14-fold increase with peptide 44. The difference in PSN-12.1 assembly inhibition potency of these two ligands indicates a positional effect of peptide-ligand binding to the MDM2 dimerization interface in preventing dimerization. As a control, the binding of peptides 43 and 44 to MDM2 using ELISA demonstrated no effect on N-terminal-binding activity (Fig. S9B). These data highlight the difference between a standard ligand-binding assay that does not distinguish between the oligomeric or monomeric nature of the target protein (Fig. S9) and a SERS-based ligand-binding assay that requires target protein “oligomerization” (Fig. 6; PSN assembly model illustrated in Fig. 4A, i and ii).

Discussion

A number of SERS-based immunoassays have been developed for the detection of biological interactions; however, few NP–protein aggregation studies have been published. This report presents the use of protein–peptide interactions as a controlled NP–assembly template capable of “turning on” SERS. Furthermore, the protein interactions investigated are of particular biological interest owing to the critical role of MDM2 in cancer progression. PSN assembly and associated SERS enhancement were successfully inhibited by preincubating MDM2 with small-molecule-binding ligands.

We have demonstrated that full-length MDM2 is able to successfully aggregate PSN-12.1, suggesting that the MDM2 protein is in dimeric (oligomeric) state in solution, while maintaining biological activity of the hydrophobic pocket. These studies present an innovative method for interrogating the allosteric interactions of full-length unlabeled MDM2 using biologically driven NP assembly. We also demonstrate a proof-of-concept with which to use SERS-based ligand-binding assays to investigate other allosteric proteins that undergo complex conformational interactions.

Methods

Peptide–BT Conjugation. Peptides 12.1 (SGSG-MPRFMDYWEGLN-resin) and 12.1(W8A) (SGSG-MPRFMDYAEGLN-resin) were obtained bound to Wang resin via the C terminus (Almac Sciences), with the N terminus deprotected. Modification with BT was carried out via amide coupling in the solid phase and cleaved from the resin using 95% TFA (SI Methods).

Nanoparticle Bioconjugation. AgEDTA nanoparticles were synthesized with a 40-nm diameter using the method described by Heard et al. in 1983 (52). Nanoparticles were centrifuged at 1,900 × g for 20 min and resuspended in buffer (25 mM Hepes and 20 mM KCl (pH 7.5)) before addition of BT or peptide–BT at a final concentration of 10^−9 M. After shaking for a minimum of 1 h, the conjugation solutions were centrifuged at 5,000 rpm for 20 min and resuspended in buffer (pH 3) to remove any excess analytes. Complete BT–peptide conjugates were characterized using extinction spectroscopy, dynamic light scattering, and MALDI-MS.

Dynamic Light Scattering. One-milliliter samples were analyzed via dynamic light scattering using a Malvern high-performance particle sizer (HPPS) using standard disposable cuvettes.

Extinction Spectroscopy. Absorbance readings were taken from 250–650 nm using a Cary Eclipse extinction spectrometer. All spectra were baseline corrected using 25 mM Hepes buffer (pH 7.5), 20 mM KCl, as a blank. NP concentrations were calculated using the extinction coefficient for 40-nm silver nanoparticles at λ-max (ε = 2.87 × 10^4 M). Peptide–BT concentrations were calculated using the extinction coefficient for BT at 487 nm (ε = 12017).

MALDI-MS. Peptide samples were analyzed using a 1:1 ratio of sample to matrix, α-cyano-4-hydroxycinnamic acid (α-cyano). Ionization was conducted in the positive reflectron mode. A linear three-point calibration was achieved using a preprepared peptide mixture: 379.1 m/z (α-cyano matrix), 757.4 m/z (Bradykinin fragment), and 1,046.5 m/z (angiotensin II). To analyze nanoparticle-bound peptide samples, conjugates were removed from the nanoparticles and desalted. Nanoparticle samples were treated with DTT (10 mM) for a minimum of 30 min to displace BT from the metal surface. Following centrifugation at 5,000 rpm for 20 min, 150 μL of supernatant was desalted using PepClean C-18 spin columns (Thermo Scientific) MALDI-MS carried out on the recovered sample.
MMD2 was purified as indicated in SI Methods. Purified full-length MMD2 was stored at −20 °C in storage buffer (25 mM Hepes (pH 7.5), 10% (v/v) glycerol, 1 mM benzamidine, 5 mM DTT, 200 mM KCl, and buffer exchange into assay buffer (25 mM Hepes (pH 7.5), 20 mM KCl) was performed using a concentrator with a 10-kDa molecular mass cutoff filter (Millipore). BSA was obtained from Sigma and dissolved in assay buffer. MDM2 and BSA stocks were prepared at a concentration of ∼8 × 10^−9 M in assay buffer for use in NP aggregation assays. MDM2 protein was validated in the dual-site-binding assay that measures the ability of Nutlin to stimulate the interaction of MDM2 with the Rb1 peptide (10). Rb1 peptide binds to the acidic domain of MDM2 and mimics the interaction of MDM2 with the p53 central DNA-binding domain (10).

SERs Analysis. SERs spectra were collected using a Renishaw iVia microscope system. Excitation at 514.5 nm was achieved via an Ar+ laser attenuated using neutral density filters. Spectra were obtained using 180° backscattering with the grating centered at 1400 cm−1 using a 20× long-working distance objective. Static scans with a 2-s collection time were obtained for NPs adsorbed on the nanomaterials in a droplet containing buffer. Three independent analyses of samples was conducted using an NMR tube spinner microscope attachment. A 1-s scan duration was used, and one spectrum was acquired every 60 s or 30 min.

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Supporting Information

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SI Methods

Peptide Modification and Nanoparticle Conjugation. Peptides 12.1 and 12.1$_{WAA}$ with an N-terminal GSGS spacer, were purchased attached to Wang resin via the C terminus. The N terminus was deprotected ready for modification with BT in the solid phase, as shown in the schematic (Fig. S1). Full sequences (C–N) of peptides 12.1 and 12.1$_{WAA}$ are as follows: Resin-NLGE-WYDMFRPM-GSGS and Resin-NLGEAYDFRPM-GSGS, respectively. Peptide was calculated to account for 70% of the total peptide–resin weight, and peptide-bound resin (12.14 mg of 12.1, 19.4 mg of 12.1$_{WAA}$) was preswelled in 1 mL of dimethylformamide (DMF) for a minimum of 30 min. A total of 1.5 eq (1.75 mg 12.1, 3.04 mg 12.1$_{WAA}$) O-(7-Azabenzotriazol-1-yl)-N,N,N,N-tetramethyluronium hexafluorophosphosphate (HATU) and 3 eq (1.70 μL 12.1, 4.40 μL 12.1$_{WAA}$) N,N-disopropylethylamine (DIPEA) were mixed with 1 eq BT (153.4 μL 12.1, 175.3 μL 12.1$_{WAA}$ of 2 × 10$^{-2}$ M in DMF) and immediately added to the preswelled peptide–resin. The reaction mixture was left to stir at 20 °C for 6 h. Following removal of the reaction solution, resin was washed once with DMF (1 mL), acetonitrile (MeCN) (3 mL), and diethyl ether (DEE) (1 mL), and immediately added to the dried resin. After stirring for 16 h, the solution was discarded and resin was washed with DMF (1 mL), MeCN (3 mL), and DEE (3 × 1 mL) as described above. Modified peptides were cleaved from the resin using a 2.2-mL cleavage mixture [2 mL trifluoroacetic acid (TFA), 100 μL triisopropylsilane (TIS), and 100 μL d$_2$H$_2$O] for 4 h in an inert atmosphere. Cleaved peptide was filtered from the resin using a 1.0-μm polystyrene–fluoroethylene (PFTFE) filter, which was washed through with MeCN (2 × 2 mL). Solvent was removed under reduced pressure, and cold DEE (1 mL) was added to afford a precipitate. Following DEE removal, the precipitate was dissolved in an appropriate volume of MilliQ-H$_2$O (4 mL). Unmodified peptides 12.1 and 12.1$_{WAA}$ were prepared by preswelling the resin for a minimum of 30 min in DMF and cleaving from resin as described for peptide–BT conjugates. Peptide 12.1 is reported to form an α helix, and this was verified by CD in 50% Trifluoroethanol (TFE). CD results showed peptide 12.1 to have a random conformation in aqueous solution with the propensity to adopt a helical secondary structure (Fig. S24). Analysis also demonstrated that the potential for peptide 12.1 to form a helical conformation is not hampered by BT conjugation (Fig. S2B). Peptide 12.1–BT and mutant peptide 12.1$_{WAA}$ demonstrated high- and low-binding affinity for the MDM2 hydrophobic cleft in a competition ELISA (Fig. S3). Nanoparticle conjugates were characterized by extinction spectroscopy and MALDI-MS. A red shift, of ~4 nm, of the plasmon band λ-max monitored by extinction spectroscopy (Fig. S4) shows an increase in particle size relative to the size of conjugating molecule. The red shift is more pronounced for peptide 12.1 than for peptide 12.1$_{WAA}$ because of the presence of tryptophan.

Successful modification of peptides 12.1 and 12.1$_{WAA}$ with BT was shown by MALDI-MS analysis (Fig. S5). Ions with $m/z$ 563.9, 1,845.9, and 2,391.9 represent BT peptide, 12.1$_{WAA}$, and peptide 12.1–BT, respectively, whereas values of 1,730.7 and 2,276.3 $m/z$ correspond to peptide 12.1$_{WAA}$ and peptide 12.1$_{WAA}$–BT. Following nanoparticle conjugation, analytes were removed from the nanoparticle surface using DTT displacement. MALDI-MS analysis of the removed analytes was used to verify presence of the peptide on the nanoparticle surface.

Characterization of PSN Assembly. SEM analysis of PSN samples with and without MDM2 shows partial aggregation of PSN-12.1 following MDM2 addition (Fig. S6). Formation of predominantly dimers and trimers provides an explanation for the small changes observed in extinction spectroscopy compared with a relatively large increase in SERS intensity.

MDM2 was replaced by BSA in the reaction mixture as an alternative control to test the biological specificity of the interaction. Extinction spectroscopy and SERS results are shown for PSN samples with a 1:1,000 ratio of PSN:protein (Fig. S7).

MDM2 Purification, MDM2 Ubiquitination Assays, and Protein-Binding ELISAs. Bacterially expressed full-length, untagged human MDM2 was expressed in Escherichia coli BL21 cells harboring a pT7.7 plasmid containing the MDM2 gene. The MDM2 protein was induced by addition of 1 mM final concentration of isopropyl-l-thio-d-galactopyranoside and 100 μM zinc chloride. Sedimented cells were washed with 50 mM Tris-HCl (pH 8.0), and the final pellet was resuspended to OD. 100 (optical density at 595 nm) in 10% (vol/vol) sucrose, 50 mM Tris-HCl (pH 8.0), lysozyme (150 μg/mL), Pefabloc (2 mM; Roche Molecular Biochemicals), DTT1 (5 mM; BDH Laboratory Supplies), benzamidine (1 mM), and NaCl (0.25 M final concentration). The cell suspension was incubated on ice 45 min, 37 °C for 1 min, and then returned to ice. The cells were lysed on ice by sonication and the lysate was centrifuged and filtered through a 0.22-μm Whatman syringe filter, and the supernatant was fractionated on a 5-mL HiTrap-SP column (Amersham Biosciences) equilibrated with buffer A [15% glycerol, 25 mM Hepes (pH 8.0), 0.02% Triton X-100, 5 mM DTT, and 1 mM benzamidine]. The supernatant was diluted 5× with buffer A before application onto the column, and bound protein was eluted in a 40-column volume linear gradient in buffer A from 0.05 to 1.0 M KCl. Aliquots of the fractions from the column were assayed for the purity of MDM2 by SDS gel, by Western blotting, and by specific activity. Purified MDM2 (SP fractions 25–29) was used in SERS binding. Bacterially expressed MDM2 protein was also further validated in ubiquitination assays (Fig. S8C), tetrameric p53 protein binding assays (Fig. S8A), and BOX-I p53 peptide-binding assays (Fig. S8B) to demonstrate that the 12.1 peptide used in SERS was bioactive in standard MDM2 activity assays.

To measure the binding of MDM2 protein to peptides or p53 in the solid phase by ELISA, 96-well plates (Costar) were coated with p53 protein (20 ng) in coating buffer [0.1 M NaHCO$_3$ (pH 8.6)] by incubation overnight at 4 °C. For p53–peptide binding, the BOX-I peptide (1 ng/well) was added to an ELISA well precoated with streptavidin in coating buffer [0.1 M NaHCO$_3$ (pH 8.6)] (500 ng/well) and prewashed with PBS-T (PBS supplemented with 0.1% Tween-20); nonspecific binding was blocked by incubation in blocking buffer (3% BSA in 1× PBS) for 1 h at room temperature. After either p53 adsorption or p53–peptide capture, and washing with PBS-T (PBS supplemented with 0.1% Tween-20), nonspecific binding was blocked by incubation in blocking buffer (3% BSA in 1× PBS) for 1 h at room temperature. A titration of MDM2 was performed (without or with preincubation with ligands; Figs. S3 and S9), and the plate was incubated for 1 h at room temperature. Following further washes, the plate was incubated with primary antibody specific to MDM2 for 1 h at room temperature. After washing, the ap-

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propriate HRP-conjugated secondary antibody was added and the plate was incubated for 1 h at room temperature. After final washing, binding was measured by enhanced chemiluminescence (ECL). The luminescence produced was immediately detected with a Fluoroskan Ascent FL luminometer and Ascent software version 2.4.1 (Labsystems).

![Diagram](image.png)

**Fig. S1.** Schematic of peptide-BT modification procedure where R can represent either peptide 12.1 or peptide 12.1\textsubscript{WLA}. 

**Fig. S2.** (A) Far UV CD spectra of unmodified peptide 12.1 in H$_2$O (pink) in 10% TFE (blue) and 50% TFE (green). (B) Far UV CD spectra of peptide 12.1-BT in H$_2$O (pink) and 50% TFE (blue).

**Fig. S3.** Competitive inhibition of MDM2-binding to p53-derived BOX-I peptides by ligands. Competitive ELISAs that measure the binding between full-length MDM2 and p53-derived BOX-I peptide ligand were performed as described previously (1). Biotinylated p53-BOX-I peptide was adsorbed to a streptavidin-coated ELISA well, and fixed levels of MDM2 were added into a buffer with increasing amounts of the indicated ligand; peptide 12.1, mutant peptide 12.1$_{WAA}$, and Nutlin-3. Peptide 12.1 and mutant peptide 12.1$_{WAA}$ are represented by F and CFA respectively. Reaction solutions were added to the ELISA well and incubated for 1 h at room temperature. Binding of MDM2 was quantified using an anti-MDM2 MAb and secondary antibody coupled ot peroxidase, as indicated (1). The data demonstrate that peptide 12.1 is almost as potent as Nutlin-3 in displacing MDM2 from p53.


**Fig. S4.** Extinction spectroscopy of AgEDTA conjugates. Plasmon band £-max of conjugates are 416 (AgEDTA), 421 (PSN-12.1), and 419 (PSN-12.1$_{WAA}$).
Fig. S5. MALDI-MS analysis [entire spectra (A) and magnified regions (B)] of peptide 12.1–BT (purple), peptide 12.1–BT removed from nanoparticles (yellow), peptide 12.1\(\Delta\)A–BT (green), peptide 12.1\(\Delta\)A–BT removed from nanoparticles (blue) and BT removed from nanoparticles (red).
(A) SEM images of PSN-12.1 (i), PSN-12.1-MDM2 (ii), PSN-12.1_WΔA (iii), and PSN-12.1_WΔA-MDM2 (iv). (B) Statistics from SEM analysis showing the percentage of different size clusters. Numbers in the legend represent the number of PSNs per cluster.

Fig. S6.
Fig. S7. Extinction spectroscopy (A) and SERS analysis (B) of PSN before and after the addition of MDM2/BSA.

Fig. S8. Validation of MDM2 protein-specific activity. (A) p53 protein binding. Tetrameric full-length p53 was bound to the solid phase, and the indicated amounts of MDM2 protein were titrated into ELISA wells to measure the activity of MDM2 protein. The data are plotted as MDM2-binding activity toward p53 (in relative light units) as a function of MDM2 titration. (B) p53-BOX-I peptide binding. The biotinylated p53 peptide was captured on streptavidin-coated ELISA wells, and the indicated amounts of MDM2 protein were titrated into ELISA wells to measure the activity of MDM2 protein. The data are plotted as MDM2-binding activity toward p53 (in relative light units) as a function of MDM2 titration. (C) MDM2 activity as an E3 ubiquitin ligase. Reactions contained 25 mM Hepes (pH 8.0), 10 mM MgCl2, 4 mM ATP, 0.5 mM DTT, 0.05% (vol/vol) Triton X-100, 0.25 mM benzamidine, 10 mM creatine phosphate, 3.5 units/mL creatine kinase, ubiquitin (2 μg), E1 (50–200 nM), E2 (0.1 μM), p53 (100 ng), and human MDM2 (50 ng). Reaction products were analyzed by immunoblotting for changes in p53 mass, and the ubiquitin adducts can be observed by the multiple bands (from left lanes 3–5 vs. 1, 2, and 6).
Fig. S9. Illustration of the dimerization interface peptide interactions and verification that these interactions do not affect N-terminal-binding activity. (A) The structure of the dimeric MDM2/MDM2 RING domain [Protein Data Bank (PDB) code 2HDP] is depicted, with stabilizing interdomain interactions highlighted from the dimer interface of the MDM2/MDMX heterodimer (PDB code 2VJF) (1). One interface peptide (peptide 44; PLNAIEPCVICQGRP) in yellow (arrow) and the N terminus of the junction interface (peptide 43; DKEESVESSLPLNAI) in red (arrow) of which the latter has its N terminus outwith the structure. (B) Competitive ELISA used to measure the binding between full-length MDM2 and peptide 12.1 (2). Biotinylated peptide 12.1 was adsorbed to a streptavidin-coated ELISA well, and fixed levels of MDM2 were added into a buffer with increasing amounts of the indicated ligand: Nutlin-3, RING-binding peptide 43, and RING-binding peptide 44. Reaction solutions were added to the ELISA well and incubated for 1 h at room temperature. Binding of MDM2 was quantified using an anti-MDM2 MAb and secondary antibody coupled to peroxidase, as indicated (2). The data demonstrate that RING-binding peptides 43 and 44 do not interfere with MDM2 N-terminal binding to peptide 12.1.