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Nanoparticle Self-Assembly Directed by Antagonistic Kinase and Phosphatase Activities**

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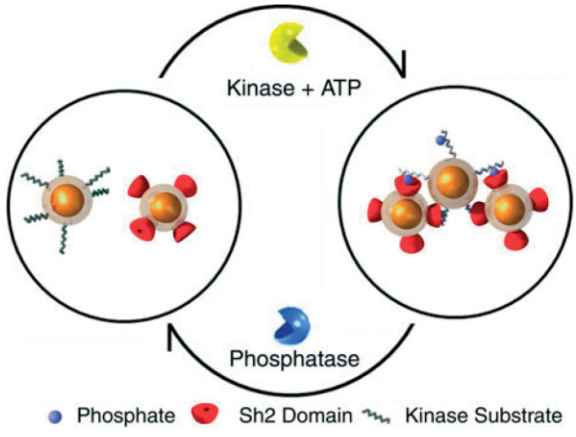
Reversible self-assembly is ubiquitous in Nature. The ability to dynamically control the assembly and disassembly of biomolecular complexes is essential to higher-order biological processes, including the control of intracellular communication,[1] gene expression,[2,3] and platelet aggregation upon injury.[4] To date, assembly or disassembly of nanoparticles (NPs) has been exploited to improve sensitivity for detection of single biomolecular targets, including DNA,[5,6] small molecules, proteins,[7,8] and pH changes[9] in vitro; however, mechanisms[9b] of sensing that allow multiple, opposing stimuli to dynamically assemble *and* dis-assemble NPs have not been described. Development of inorganic nanoparticles that respond to multiple, antagonistic biological signals could facilitate sensing of the physiologic balance between opposing effectors of cellular and tissue function. Herein, we introduce a nanoparticle (NP) system where self-assembly is dynamically coupled to the balance between the classic antagonistic enzymes: tyrosine kinase and phosphatase. In vivo, these enzymes regulate cellular communication, gene expression, and ultimately cell life and death through the phosphorylation and dephosphorylation of tyrosine residues on other proteins.[10,11] Their dysregulation contributes significantly to the development of cancer and other inflammatory diseases.[12,13] Here, kinase-induced superparamagnetic nanoassemblies enhance the T2 relaxation of hydrogen atoms at picomolar enzyme concentrations and are shown to be fully reversible by introducing excess phosphatase activity. In the future, these nanomaterials may be optimized to report the balance between these cytosolic enzyme activities and may facilitate new screens for inhibitors in vitro and in vivo. Additionally, extensions of this design logic may be synthesized to probe the dynamics of a diversity of antagonistic biologic processes.

To construct a system of NPs that could coalesce in the presence of kinase activity and re-disperse in the presence of phosphatase activity, two NP populations were synthesized (Scheme 1). The first population was modified with peptide substrates that may be phosphorylated by Abl tyrosine kinase and dephosphorylated by a phosphatase. The second population was modified with Src Homology 2 (SH2) domains that recognize and bind the phosphorylated Abl kinase substrate in a sequence-specific manner.[14] Together, these NPs process kinase and phosphatase activities by assembling as peptides become phosphorylated and disassembling as phosphates are removed. Magnetic dipoles in NP assemblies coordinate and more efficiently dephase hydrogen protons in MRI, allowing T2 relaxation mapping of kinase function.[15,16] Conceptually, this design is akin to the kinase/phosphatase FRET sensors



Scheme 1. Polymer-coated, superparamagnetic NPs were modified to polyvalently display either a tyrosine-containing kinase substrate or an SH2 domain. NPs remain dispersed until kinases phosphorylate substrate NPs, triggering NP assembly via phosphopepetide-SH2 binding. Kinase-directed assembly amplifies the T2 relaxation in MRI and is fully reversible by phosphatise.

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developed by Tsien et al[17–19] among many other fluorescence-based kinase sensors,[20–23] but instead of transducing enzyme activites into optical fluorescence changes, activity is encoded via NMR relaxation changes.[16,24,25] Recently, two gold NP-based approaches have sensed either kinase or phosphatase activity in irreversible, two-step assays.[26,27] These designs provide new avenues for colorimetric screening of enzyme inhibitors, yet lack the capacity to continuously analyze both kinase and phosphatase balance. Similarly, while NP-based sensing of single analytes and enzymes has been demonstrated,[15,16,28,29] the extension of this technology to reversibly sensing multiple enzyme activities has not been accomplished.

We first set out to verify that phosphopeptide-SH2 domain binding could efficiently induce NP assembly at particle concentrations relevant to MR imaging. Dextran-coated iron oxide NPs were synthesized, cross-linked, and aminated according to published procedures.[30–32] The Crk SH2 domain was genetically modified to contain an (N-terminal) cysteine to allow convenient conjugation to NPs. GST-tagged cysteine-SH2 was expressed in bacteria, purified, and the GST affinity label was removed (see methods). Reduced cysteine-SH2 were conjugated to amine-NPs (~14 SH2 domains/NP) via highly flexible heterobifunctional linkers, containing 12 polyethylene oxide units (54.4 Å), to increase conformational freedom. In parallel, a phosphotyrosine (pY) sequence with low μM binding affinity to Crk SH2 (-QpYDHPNI-)[14,33] was synthesized with an N-terminal cysteine and attached to a second population of NPs using the same linker (~15 peptides/NP). When combined, these NPs rapidly assembled even at NP concentrations three orders of magnitude lower than the free peptide-SH2 affinity, as shown by the 10-fold hydrodynamic radius increase of 12 nM NPs within 15 minutes using dynamic light scattering (DLS) (Fig. 1). In the presence of 200 μM free pY peptide, assembly was inhibited. Further, SH2-NPs were able to discriminate pY-NPs from Y-NPs (unphosphorylated tyrosine: ~13 peptides/NP) and from a phosphopeptide not expected to bind to CRK SH2 (EAIpYAAPFAKKKC: ~14 peptides/NP).[14] To test the reversibility of this system, pY NPs and SH2 NPs assembly was interrupted with addition of 200 μM free pY-peptide or 2 μL of buffer (Fig. 1). While mixing shear stress had no affect on NP assembly, particles with 200 μM free peptide rapidly disassembled, dispersing over time.

The robust association of pY-NPs with SH2-NPs indicated that phospho-dependent NP assembly may provide a rapid mechanism for probing kinase activity. To begin, a kinase substrate (SRVGEEEHVYSFPNKQKSAEC) derived from paxillin was chosen for its

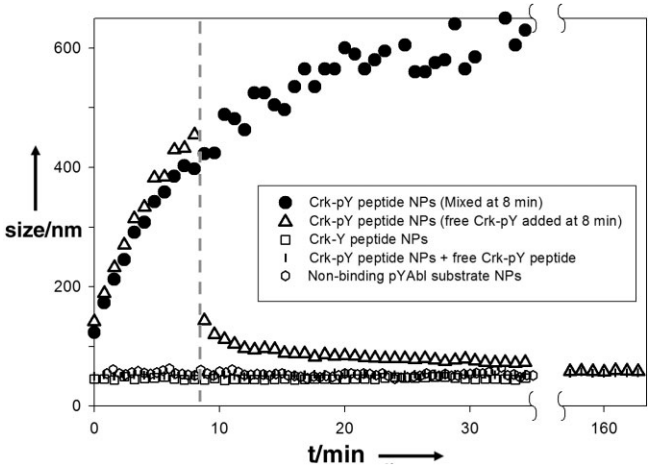

Figure 1. Phosphopeptide (pY) NP assembly with SH2 NPs. Upon addition of SH2 NPs to pY-peptide NPs, rapid increase in hydrodynamic radius was observed by DLS (●). In the presence of free pY-peptide, NP assembly was not observed (vertical lines). Non-phosphorylated peptide and non-binding pY-peptide remain dispersed with SH2 NPs, demonstrating both sequence- and phosphate- specific peptide recognition by SH2 NPs (□ and ○, respectively). Assembly was reversed by addition of excess free pY-peptide to the mixture after 8 min incubation (hollow triangles △).

Crk SH2 binding and specificity to Abl.[34,35] Three versions of this peptide substrate were synthesized and attached to NPs: a phosphorylated substrate (pY-Abl) (~11 peptides/NP), an unphosphorylated substrate (Y-Abl) (~10 peptides/NP), and a substrate where the receptor tyrosine was replaced with a phenylalanine (F-Abl) (~10 peptides/NP). Abl kinase rapidly directed assembly in solutions containing Y-Abl NPs with SH2 NPs, while an F-Abl peptide control remained dispersed in DLS (Fig. 2a). We next probed the ability of NP self-assem-

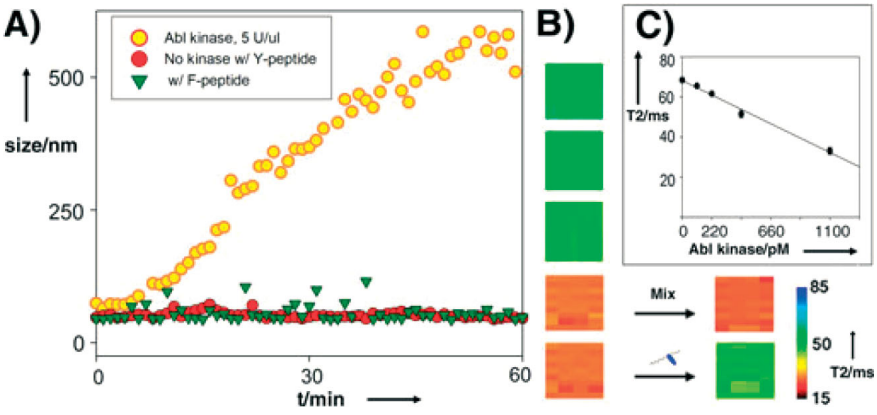

Figure 2. Kinase-directed NP assembly. A) Abl kinase (5 U μL^{-1}) (yellow dots) was added to a mixture of SH2 NPs and tyrosine-containing, Abl substrate NPs at 2 min and NP radius was observed over time using dynamic light scattering (DLS). Controls without kinase (red dots) with phenylalanine-Abl substrate NPs (green triangle) did not assemble. B) In MRI, T2 relaxation was enhanced by Abl kinase-directed assembly (bottom two wells) and was reversed by addition of 200 μM free phosphopeptide, but not by mixing alone. Controls lacking enzyme (top), containing phenylalanine substrate NPs (second from top), or 200 μM free pY substrate (third from top) did not show enhancement. C) Dose dependent T2 relaxation enhancement of SH2 NPs and Y-peptide NPs 3 h following Abl kinase addition (12 nM NPs).

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bly to transduce kinase activity into NMR T2 relaxation changes using a 4.7T Bruker MRI (Fig. 2b and c). Quantifiable T2 relaxation enhancements in solutions containing Y-Abl NPs with SH2 NPs were observed in the presence of as little as 7 femtomoles of added kinase (110 pM kinase concentrations = 0.05 U μL^{-1}) (Fig. 2c). Further, T2 enhancement was lost upon addition of free pY-Abl substrate, demonstrating that kinase-directed NP assembly depended on phosphopeptide-SH2 domain interactions that were reversible by competition (Fig. 2b).

As NPs coalesce, tyrosine-linked phosphates become sequestered in SH2 domain binding pockets. Having demonstrated that addition of free pY-peptide was able to reverse NP binding, we sought to examine whether phosphatase activity could oppose kinase-directed self-assembly by removing phosphates from tyrosine residues as they dynamically disassociate with SH2 domains. To begin, we tested YOP phosphatase's ability to counteract the rapid association of pY-Abl NPs with SH2 NPs. Similar to the competition test performed previously (Fig. 1a), assembly of pY-Abl NPs with SH2 NPs was interrupted and reversed by addition of phosphatase, but not by buffer alone (Fig. 3a). Additionally, when Y-Abl NPs and SH2 NPs were first exposed to Abl kinase and subsequently to an excess of antagonistic phosphatase, kinase-catalyzed NP assembly was efficiently reversed by the excess phosphatase (Fig. 3b and c).

Together, these results illustrate the ability of this NP system to continuously sense cycling kinase/phophatase activities in MR. Importantly, phosphatase is able to both halt NP assembly (by removing phosphates from free NPs) and to deconstruct phospho-dependent nanoassemblies (by removing phosphates as they dynamically disassociated with SH2 domains). We believe the rapid reversal of NP assembly (either via monomer competition or substrate de-phosphorylation) and the enhancement of NP avidity over anticipated monovalent binding (assembling at NP concentrations 1000-fold below peptide/SH2 affinities) are indications of polyvalent NP binding.[36] Unlike monovalent interactions, the disassociation rate of polyvalent species is accelerated by the presence of monomeric competitor.[37] Synthetically, polyvalency has been exploited to develop improved biological inhibitors,[36] targeting agents,[38,39] and affinity chromatography procedures.[37] Here, we exploit polyvalent binding to engineer a reversible NP system that assembles into stable nanostructures, yet may also be rapidly disassembled by competition.

In summary, we present the design and synthesis of a NP system that processes two antagonistic enzymes inputs (tyrosine kinase/phosphatase) to output enhanced T2 relaxation in the presence of net kinase activity. Phosphopeptide-directed assembly enables rapid MR visualization of kinase activity at nanomolar NP and picomolar kinase concentrations. To the best of our knowledge, this represents the first demonstration of a NP sensor that reversibly processes two specific, antagonistic enzyme inputs. Looking forward, as MRI field strengths increase and methods for labeling cells with nanomaterials advance,[40–42] optimizations of this design may enable a variety

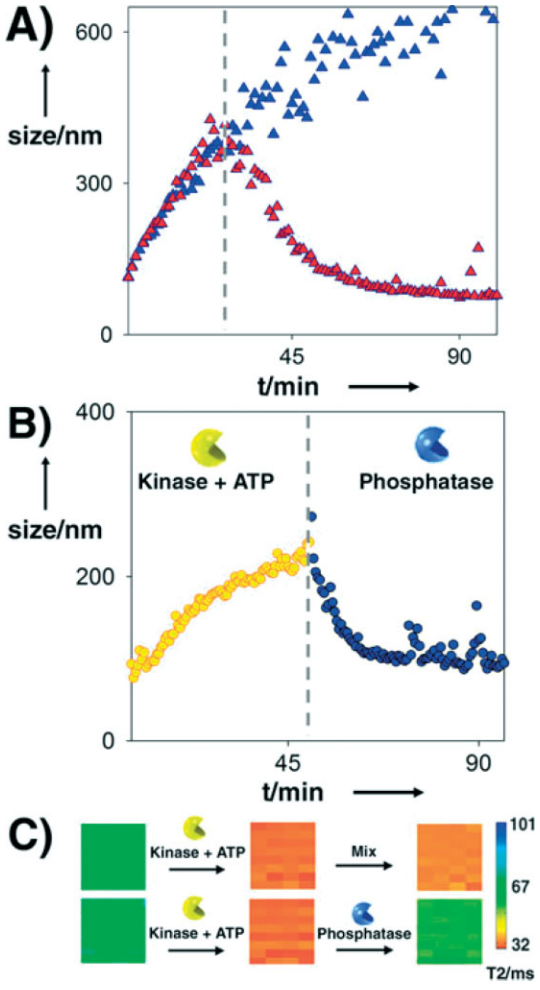

Figure 3. Phosphatase reversal of NP assembly in DLS and MRI. A) SH2 NPs and pY-Abl substrate NPs were allowed to assemble prior to addition of phosphatase (2 U μL^{-1}) (red) or vehicle control at 25 min (blue). B) NPs were exposed to Abl kinase (2.5 U μL^{-1}), followed by phosphatase (5 U μL^{-1}). C) Kinase-directed assembly (2.5 U μL^{-1}) and phosphatase disassembly (5 U μL^{-1}) was visualized via T2 relaxation enhancement in MRI.

of novel real-time assays to monitor kinase and phosphatase inhibition or to image cytosolic enzyme activities in optically opaque media and in vivo using MRI. Further, the modularity of our design should enable key features to be altered without requiring significant re-engineering. For example, this technology may be extended to incorporate new nanoparticle cores (ex. semiconductor, plasmonic) or to sense other antagonistic biological stimuli (other kinase/phosphatase pairs, acetylase/deacetylase pairs, etc.).

Experimental

Materials: All chemicals and reagents were purchased from Sigma–Aldrich unless specified. Plasmid expressing GST-Cys-SH2 was generously supplied by Dr. Barbara Imperiali (Department of Chemistry, MIT). Peptides were synthesized following standard Fmoc solid phase

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peptide synthesis method using an ABI Model 433A peptide synthesizer in MIT center for cancer research biopolymer laboratory. Nanoparticle size was measured using Zetasizer (Malvern Instruments). MRI images were taken on a Bruker 4.7T magnet. All enzyme reactions were carried out at 30 °C unless specified otherwise. Aminated nanoparticles were synthesized according to published procedures.

Expression and Purification of SH2 Domain: BL21-Gold(DE3) cells harbouring GST-Cys-Crk SH2 plasmid (pGEX4T-Cys-CrkSH2) were grown to midlog phase in LB media containing carbenicillin (50 μg mL^{-1}) at 37 °C, 220 rpm. Protein expression was induced with addition of 0.1 mM IPTG after cells were cooled to 16 °C and then, cells were incubated at 16 °C for 21 h. The cells were centrifuged at 5000 rpm, 4 °C for 30 min, and the cell pellet was resuspended in a lysis buffer (1xPBS, 100 mM EDTA, 1 % Triton X-100, 10 % glycerol, 1 mg mL^{-1} lysozyme, 1xprotease inhibitor cocktail set III (Calbiochem)) and incubated for 30 min at 4 °C. After sonication, the soluble fraction was isolated from cell debris after centrifugation (14 000 rpm, 30 min) and purified using glutathione sepharose 4B affinity column (Amersham Biosciences) following manufacture's protocol. Eluted proteins were dialyzed with 7 kD MW cutoff dialysis cassette (Slide-a-Lyzer, Pierce) against 1xPBS and characterized by SDS-PAGE. To remove GST tag, protein solution (1 mg mL^{-1}) was treated with TEV protease (50 units mL^{-1}, Invitrogen) in a TEV protease buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 8.0) in the presence of 1 mM DTT. After 4 h incubation at 25 °C, the cleavage reaction mixture was subject to glutathione column and then, Ni^{+2}-NTA column to sequentially remove cleaved GST tag and TEV protease, respectively. To ensure that cysteine thiols of cys-SH2 domain were fully reduced, cys-SH2 domain was passed through reducing column (Reduce-Imm Immobilized Reductant Column, Pierce) following manufacture's instruction immediately prior to nanoparticle conjugation.

Preparation of Peptide-Presenting Nanoparticles and SH2-Conjugated Nanoparticles: First, maleimide-activated NPs were prepared by conjugating NHS-PEO_{12}-maleimide (succinimidyl-[(*N*-maleimidopropionamido)-dodecaethyleneglycol] ester, Pierce) to aminated NPs. Typically, NPs (0.25 mg Fe) were incubated with 4 mM of NHS-PEO_{12}-maleimide for 30 min at 25 °C and then purified using a magnetic field filtration column (Miltenyi Biotec). SH2 conjugated particles were prepared by incubating Cys-SH2 (1 mg mL^{-1}) with maleimide presented NPs (0.25 mg Fe) for 3 h at RT. Unreacted Cys-SH2 domain was removed using a magnetic field filtration column. Peptides were conjugated by activating amine-NPs with NHS-PEO_{12}-maleimide as above, followed by addition of peptide substrate. Particles were filtered 2 h after peptide addition. The peptides used in this investigation were synthesized as follows:

CRK SH2-binding:

TAMRA-C(Ahx)QpYDHPNI-$CONH_2$
TAMRA-C(Ahx)QYDHPNI-$CONH_2$

Non-Binding Abl substrate:

TAMRA-(Ahx)EAIpYAAPFAKKKC-$CONH_2$

CRK SH2-binding Abl substrates:

TAMRA-(Ahx)SRVGEEEHVpYSFPNKQKSAEC-$CONH_2$
TAMRA-(Ahx)SRVGEEEHVYSFPNKQKSAEC-$CONH_2$
TAMRA-(Ahx)SRVGEEEHVFSFPNKQKSAEC-$CONH_2$

(Ahx:aminohexanoic acid)

Quantification of Peptide/SH2 Numbers per NP: The yield of the SH2 domain-NP reaction was quantified by performing a bicinchroninic acid protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) on modified versus unmodified NPs after purification. SH2 NPs were compared with their parent PEO-NPs containing a gradient of albumin. 220ug of SH2 domain (~13 kDa) were conjugated per 1 mg of Fe in NPs (805 000 mg Fe/mole NPs). Peptide attachment yields were quantified by monitoring the increase in purified NP absorbance at 555 nm following TAMRA-peptide conjugations (TAMRA extinction: 90 000 M^{-1} cm^{-1}).

SH2-Binding Peptide-Mediated Nanoparticle Assembly: Nanoparticles presenting CRK SH2-binding peptide (either phosphorylated or unphosphorylated) or non-binding Abl substrate (phosphorylated) were incubated with CRK-SH2 NPs at 10 μg Fe/mL (12 nM NP concentration) and monitored with DLS over time.

Kinase-Directed Nanoparticle Assembly: Nanoparticles presenting kinase substrate peptide (10 μg Fe/mL; 12 nM) and SH2-presented nanoparticles (10 μg Fe/mL; 12 nM) were mixed in a kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 20 mM NaCl, 0.2 mM EGTA, 0.4 mM DTT, 0.004 % Brij 35, 0.2 mM ATP) in a total volume of 50 μL. Kinase reaction was initiated by adding indicated amount of Abl kinase (New England Biolabs). NP assemblies were characterized by DLS over time or MRI.

Phosphatase-Directed Nanoparticle Disassembly: First, SH2 nanoparticles (5 μg Fe/mL; 6 nM) were added to phosphorylated tyrosine containing peptide nanoparticles (5 μg Fe/mL; 6 nM) in a buffer solution (20 mM Tris-HCl pH 7.5, 20 mM NaCl, 0.4 mM Na_2EDTA, 2 mM DTT, 0.004 % Brij 35) to initiate nanoparticle assembly. YOP protein tyrosine phosphatase (New England Biolabs, 2 U μL^{-1}) was added when size of assembled nanoparticles reached to about 400 nm in radius.

Reversal of Kinase-Induced Nanoparticle Assembly by Phosphatase: First, nanoparticle assembly was initiated following same protocols described above. Then, YOP phosphatase (5 U μL^{-1} in final concentration) was directly added into a kinase reaction mixture. Size measurement was restarted right after thoroughly mixing the reaction mixture.

MRI Imaging of Nanoparticles: All nanoparticle solutions were prepared in final concentration of 10 μg Fe/mL (12 nM) in 70 μL of kinase reaction buffer. Nanoparticle mixtures were incubated at 30 °C for 3 h after kinase additions (0, 0.05, 0.1, 0.2, 0.5 U μL^{-1}) and then, MRI images were taken using a 4.7T Bruker magnet (7 cm bore) using T2-mapping Carr-Purcell-Meiboom-Gill pulse sequence. To reverse the assembly, YOP phosphatase (4 U μL^{-1}) or free pY-peptide (0.1 mM) was added to an assembled nanoparticle solution containing 0.2 U μL^{-1} Abl kinase. The MRI image was taken after 10 min incubation at room temperature.

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