Published in final edited form as:
*ACS Nano*. 2012 May 22; 6(5): 3670–3676. doi:10.1021/nn301869z.

# Aptamer-Functionalized Nanoparticles for Medical Applications: Challenges and Opportunities

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## Abstract

With advances in aptamer selection technologies and nanomedicine, aptamer-functionalized nanoparticles are being explored as promising platforms for targeted therapeutic and diagnostic applications. In this Perspective, we outline recent progress in this field, as exemplified by Bamrungsap *et al.* in this issue of *ACS Nano*. Furthermore, we highlight the challenges and opportunities in translating current proof-of-concept designs into *in vivo* applications, with emphasis on the intrinsic properties of aptamers and their interplay with nanoparticles. With continuous efforts, we expect aptamer-functionalized nanoparticles to advance from preclinical into clinical development for further evaluation.

The development of aptamers as targeting ligands has passed several milestones in the past three decades. The first description of nucleic acid ligands, later named aptamers, dates back to the 1980s, when some virus-encoded small-structured RNAs were shown to bind to viral or host proteins with high affinity and specificity.[1] In 1990, the research teams of Szostak and Gold independently described a methodology, termed *in vitro* selection[2] or systematic evolution of ligands by exponential enrichment (SELEX),[3] to enrich aptamers with high-affinity binding to target molecules. Following this groundbreaking discovery, a plethora of aptamers were isolated to target molecules of interest, including some cancer-related proteins. In 2003, a selection strategy named Cell-SELEX was designed to target whole cells.[4] This strategy allows the isolated aptamers to recognize cells without prior knowledge of the target molecules. In 2006, a counter-selection process was integrated into the conventional Cell-SELEX scheme, whereby the selection process itself could differentiate different types of cells, thus making it possible to obtain cell-specific aptamers.[5] In 2010, researchers conducted an "*in vivo* selection" approach in tumor-bearing mice to isolate aptamers capable of localizing to the tumor site.[6] Toward the purpose of targeted intracellular delivery of therapeutics, a "cell-uptake selection" strategy was designed in 2011 to enrich cancer-cell-specific internalizing aptamers.[7] Taken together, the progression from isolating against simple targets to complex targets and from enriching high-affinity aptamers to internalizing aptamers, has paved the road for the development of a myriad of aptamer ligands for medical applications.

Concurrently, with the development of novel nanotechnologies for medical applications—referred to as nanomedicine—it is becoming apparent that nanomedicine may fundamentally revolutionize disease prevention, diagnosis, and treatment.[8,9] It is increasingly possible to develop nanomedicines that may: 1) improve the pharmaceutical and pharmacological

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***Conflict of Interest.*** O.C.F has financial interest in BIND Biosciences, Selecta Biosciences, and Blend Therapeutics, biopharmaceutical companies that are working to develop therapeutic nanoparticles.

properties of drugs, 2) target the delivery of drugs in a tissue- or cell-specific manner for enhanced therapeutic efficacy and safety, 3) enable the transport of drugs across a range of biological barriers including epithelial and endothelial, 4) facilitate the delivery of drugs to intracellular sites of action, 5) deliver multiple types of therapeutics with potentially different physicochemical properties, 6) deliver a combination of imaging and therapeutic agents for real-time monitoring of therapeutic efficacy, 7) bypass multidrug resistance mechanisms that involve cell-surface protein pumps (*e.g.*, P-glycoproteins), as nanoparticles (NPs) enter cells *via* endocytosis, and 8) potentially develop highly differentiated therapeutics protected by a unique set of intellectual properties.[10,11]

By integrating the advantages of nanomedicine with the cell-targeting capabilities of aptamers, aptamer-functionalized NPs may open the path to new and sophisticated design solutions for biomedical applications. In 2004, Farokhzad *et al.* reported the development of NP–aptamer conjugates for the targeted delivery of NPs into cancer cells.[12] Since then, along with the discovery of new cell-specific aptamers from the continuously developing SELEX technologies, many novel NP–aptamer platforms have been designed for high-efficiency cell sorting, targeted imaging, and targeted therapy. The detailed summary of these technologies is listed in Table 1. As an example, in this issue of *ACS Nano*, Bamrungsap *et al.* reported the use of an array of aptamer-conjugated magnetic NPs (ACMNPs) for pattern recognition of cancer cells (Figure 1).[13] In the absence of cancer cells, ACMNPs were well dispersed in the medium buffers, yielding a high T2 signal. Upon the addition of cancer cells, the binding of aptamers to their target membrane proteins results in the aggregation of ACMNPs onto the cell surface, leading to a decrease in T2 signal. Consequently, the change in the T2 signal (ΔT2) indicates a positive correlation to the protein expression level on the cell surface. Since the membrane proteins are variably expressed among different cell lines, the array of ACMNPs created a pattern recognition profile, enabling the accurate differentiation of cancer cells at the molecular and single-cell levels.

Despite some of the potential advantages of aptamers as a ligand class, the utilization of aptamers to direct NP targeting has not been widely embraced in comparison to other ligand classes, including antibody fragments and small molecules. Currently, all of the aptamer-functionalized NPs remain at the discovery or preclinical proof-of-principle stage. Among the six targeted therapeutic NPs in clinical development today, all use antibody fragments, proteins, or small molecules as targeting ligands.[11] The reason for the above phenomenon is complex and needs to be explored on a case-by-case basis. While current studies emphasize the advantages of aptamers as targeting ligands (*e.g.*, high affinity, low immunogenicity, and little variability in the production process), they may ignore the intrinsic properties of aptamers (*e.g.*, conformational flexibility, length, *in vivo* nuclease stability, manufacturing cost, intellectual property constraint, binding or internalizing features, and aptamer density on NP surface) and their interplay with NPs. Once aptamers are immobilized on the surface of NPs, the overall targeting capabilities of NP–aptamer conjugates may become distinct from the aptamers alone. In this Perspective, we highlight these factors, and discuss how the NP–aptamer interaction provides potential challenges and opportunities in the design of aptamer-functionalized NPs for medical applications (Figure 2).

## Conformational Flexibility of Aptamers

The binding of aptamers to their targets is dependent on their secondary or tertiary conformation, which in turn varies by the target's environmental conditions (*e.g.*, ionic strength, temperature, pH value, and the existence of complementary sequence). This flexible feature may be a potential barrier when using *in vitro* selected aptamers for *in vivo* applications. Because *in vitro* experimental conditions are different from *in vivo*

environments, some high-affinity aptamers obtained from *in vitro* selection would lose their effectiveness *in vivo*. To overcome this limitation, possible strategies could be to maximally mimic *in vivo* conditions during the *in vitro* selection process, or to "fix" the desired aptamer conformation prior to *in vivo* applications. Alternatively, *in vivo* selection strategies may mitigate this potential problem.[6]

Meanwhile, the flexibility of aptamer conformation provides a unique opportunity to facilitate the engineering of "smart" targeted NP platforms. In one example, researchers designed a "reversible" aptamer-functionalized liposome platform, whereby its targeted delivery capability can be antagonized by using an "antidote" molecule based on Watson-Crick base pairing.[14] Such a reversible platform could serve as a break to stop the targeting of NP–aptamer conjugates and potentially to alleviate the side effects that may be encountered from overdoses or allergic reactions to the NP–aptamer conjugates under some physiological conditions. In another example, an aptamer-functionalized single-walled carbon nanotube (SWNT) platform was engineered for "controllable" photodynamic therapy.[15] The physical wrapping of aptamer-conjugated photosensitizers around the SWNT surface inactivates the photosensitizer, which can be reactivated for therapeutic purposes in the presence of target cells. The conformationally flexible property of an aptamer differentiates it from other targeting modalities (small molecules, peptides, recombinant proteins, and antibodies). The development of such "reversible" or "controllable" targeted NP–aptamer conjugates may represent a promising platform for a myriad of medical applications.

## The Lengths of Aptamers

In the aptamer selection process, a typical oligonucleotide library consists of aptamers with relatively long lengths of ~75–100 nucleotides. These nucleotides have constant regions at both ends separated by a variable region of 25–60 nucleotides, rendering an enormous diversity of possible sequences and a vast number of different conformations with different binding properties, all of which increase the possibility of obtaining the optimal aptamer candidates.

However, the relatively long length of aptamers and the associated negative charge of the oligonucleotide phosphate backbone may represent a hurdle in creating effective targeted NPs. Negative charge on the NP surface (ζ potential) is associated with rapid clearance NPs in the liver and spleen.[16] In addition, it could increase non-specific interactions with positively charged NPs, rendering the aptamers less effective for cell targeting.[17] Aptamers of long length could create steric hindrance in the NP–aptamer chemical conjugation process, which leads to ineffective conjugation or decreased reaction yield. Furthermore, long oligonucleotides are difficult to generate at large scales by solid phase synthesis approaches. To overcome this challenge, a size-minimization step is often adopted in the post-selection process whereby the regions not critical to maintaining the binding specificity and affinity are truncated. Additionally, linkage of NPs and aptamers with a neutrally charged PEG layer could possibly mask the surface charge of NPs and decrease the NP–aptamer steric interactions.

## Nuclease Stability of Aptamers

In general, aptamers composed of unmodified nucleotides are susceptible to nuclease-mediated degradation. To stabilize aptamers, current methods more often rely on partial or complete chemical modifications, including 1) substitution of the 2' position with a fluoro, an amino, or a methoxy group, 2) replacement of internucleotide linkages with phosphorothioate linkages, and/or 3) 3'-end capping modifications. The incorporation of locked nucleic acid (LNA) modifications into aptamers or the usage of spiegelmers (*i.e.*, L-

enantiomeric oligonucleotides) has also been shown to increase their resistance to serum nucleases significantly.[18] Despite these advances, a thorough investigation of the functional mechanisms of these modifications and whether these modifications would affect the targeting capability is still needed.

Interestingly, the interplay of NPs with nucleotides may possibly protect nucleotides from nuclease-mediated degradation. The SWNT-interacted DNA strands were found to be more stable inside the cytoplasm than free DNA strands, presumably because the interaction between DNA strands and SWNTs renders the secondary structure of the DNA strand unrecognizable by the enzyme binding pockets.[19] Similarly, the gold NP–DNA conjugates are shown to be resistant to nuclease degradation. This is possibly because, as illustrated by the authors, the high surface density of DNA strands creates high local ion concentrations, thus inhibiting the attack of nucleases on the DNA structure.[20] It would be interesting to explore on a case-by-case basis whether the immobilization of aptamers on the NP surface confers the protection of aptamers from nuclease degradation. This understanding can guide the potential usage of non-chemically-modified aptamers to direct NP targeting *in vivo*.

## Manufacturing Cost and Intellectual Property (IP) Constraint

The high cost-of-goods for aptamer manufacturing is one of the main reasons for their slow pace in reaching the marketplace.[21] The relatively short shelf life of aptamers could also impact the manufacturing cost. In particular, current aptamer technologies are largely covered by several concentrated IP portfolios,[18,22] which further increases the cost and hinders more widespread aptamer research and development efforts. Consequently, despite the continuous advances in SELEX technology that have enabled the isolation of aptamers against most targets, only a few companies are engaged in aptamer development and commercialization. All these factors may provide additional challenges for engineering NP–aptamer conjugates.

It is worth noting that the cost of manufacturing aptamers has fallen over the past few years, in part due to the expiration of some original patents in this area.[18] Continuing improvements in oligonucleotide synthesis technologies would accelerate the development of cost-effective manufacturing processes in the future.

## Aptamers with Binding or Internalizing Features

When functionalizing NPs with aptamers, the choice of aptamers with either binding or internalizing features depends on the particular targeting purpose of the NPs. For targeted imaging or cell-sorting applications, binding of aptamers to the cell-surface antigens without internalization is sufficient to achieve optimal results. For drug-delivery applications, it may be desirable to use internalizing aptamers for intracellular delivery of NPs, which results in higher drug concentration inside the cells and potentially enhanced cellular toxicity. In the case of delivering siRNAs or miRNAs, which need the cytosolic environment for bioactivity, internalizing aptamers are required to facilitate the NP endocytosis process, followed by pairing with effective endosomal escape strategies to achieve maximal silencing effects. In the case of most enzyme-prodrug cancer therapy approaches, it may be important that NP internalization does not occur, which facilitates the conversion of prodrugs into active drug molecules.[23]

In a recent study, the formation of a unique nanostructure, termed “aptamer micelle,” was shown to change the aptamer feature from non-internalization to internalization.[24] The TDO5 aptamer used in the study can only bind to but not internalize into its target cells; however, the TDO5-micelle was shown to internalize into its target cells presumably by changes in the endocytosis pathways. This is a good example to demonstrate how NP–

aptamer interactions change the behavior of aptamers, and may widen the usage of some non-internalizing aptamers for targeted delivery applications.

## Aptamer Density on NP surface

The NP surface density of aptamers is a key parameter affecting the cellular binding and uptake of NP–aptamer conjugates. Due to the avidity effects, the increase in aptamer surface density significantly enhances NP binding properties[25] and cellular uptake of NP–aptamer conjugates.[16] For example, a 50 nm × 10 nm gold nanorod can be surface functionalized with approximately 80 aptamers, which renders the binding avidity of NP–aptamer conjugates 26-fold higher than the intrinsic affinity of the original aptamers.[25] This feature would improve our ability to perform cell-based imaging or targeting assays when using aptamers with relatively weak binding affinities or differentiating cells with relatively low densities of target antigens.

However, when using the NP–aptamer conjugates for tumor targeting *in vivo*, the higher aptamer surface density may not be positively related to higher tumor accumulation.[16] The presence of relatively high aptamer density on the NP surface leads to increases in the liver accumulation of NPs, thus decreasing the tumor accumulation of NPs, presumably because of excessively masking the PEG layer while concurrently adversely affecting the NP surface charge.[16] To achieve the best performance *in vivo*, the aptamer density on the NP surface needs to be precisely optimized to facilitate the balance between tissue biodistribution and cellular uptake. In addition, the high surface density of aptamers may result in intramolecular interactions among aptamers and impair aptamer conformations for cancer cell targeting, which needs to be investigated in further studies.

## Outlook

With recent scientific advances, it will be increasingly feasible to design selection strategies to isolate novel aptamers capable of recognizing disease-specific or disease-associated antigens and to use them to differentially target the diseased cells. Herein, in this issue of *ACS Nano*, Bamrungsap *et al.* demonstrated the utilization of aptamers isolated using the Cell-SELEX strategy to construct NP–aptamer conjugates for pattern recognition of cancer cells.[13] When designing these constructs, it is important to consider the “aptamer-functionalized NPs” as an integrated entity with a unique set of properties that may be distinct from NPs or aptamers. We anticipate selection strategies that may utilize NP-oligonucleotides libraries in the screening process to yield desirable NP–aptamer conjugates.

With the early concentrated aptamer IP portfolios, including the foundational aptamer discoveries, beginning to lose exclusivity, the door may open for more development and commercialization efforts in this field. We expect to see the continued emergence of newer NP–aptamer technologies that take advantage of aptamers’ characteristics for enabling unique functional properties. Meanwhile, we recognize that nearly all classes of nucleic acid therapeutics, includinganti-sense oligonucleotides, genes, siRNAs, and aptamers, have historically faced development challenges, in large part because of delivery issues. Therefore, we are optimistic, albeit cautiously, regarding the aptamer global market, which is estimated to grow from $236 million in 2010 to nearly $1.9 billion in 2014.[26] These are exciting times for both aptamer and nanomedicine research, and the pace of scientific discoveries is gaining momentum in these fields.

## Acknowledgments

This work was supported by National Institutes of Health Grants CA151884 and EB003647, the David Koch—Prostate Cancer Foundation Award in Nanotherapeutics.

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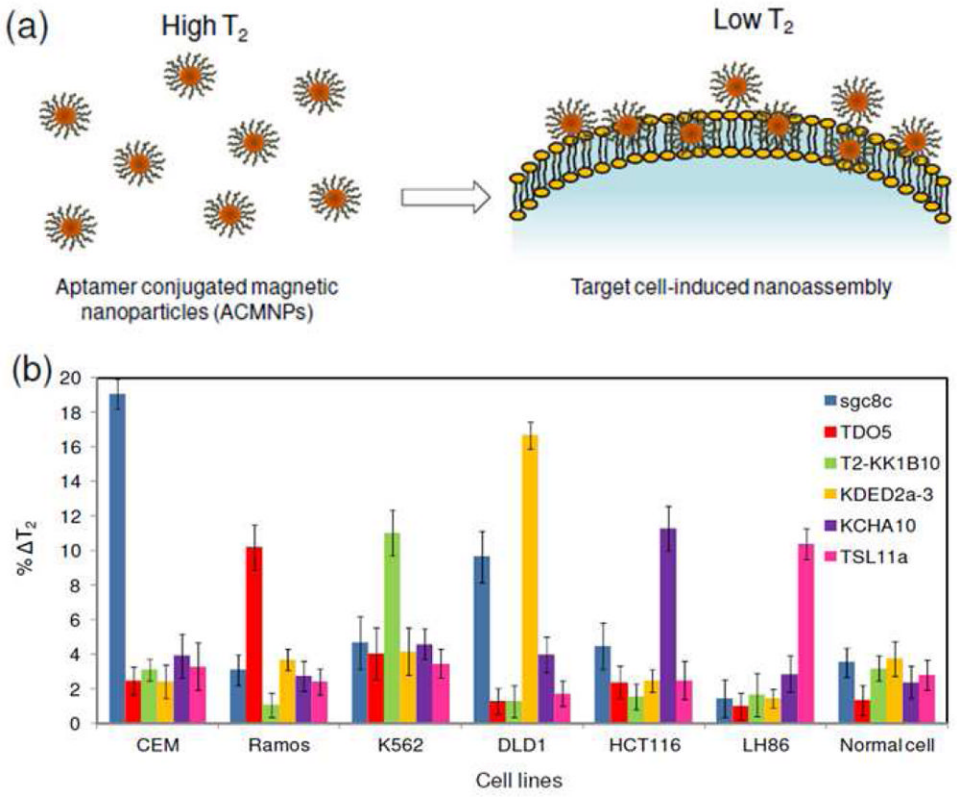


**Figure 1.**

(a) Schematic illustration of using the ACMNPs for cancer cell detection. ACMNPs were well dispersed in the medium buffers, yielding a high T2 signal without the existence of target cells. Upon the addition of target cells, the binding of aptamers to their targeted membrane proteins results in the aggregation of ACMNPs onto the cell surface, leading to a decrease in T2 signal. (b) The use of ACMNPs for pattern recognition of cancer cells. The % ΔT2 was obtained by incubating different ACMNPs with various target cancer cell lines or control normal cell lines. All the measurements were performed using 1000 cells in 250 uL sample volume. Reprinted from ref 13. Copyright 2012 American Chemical Society.

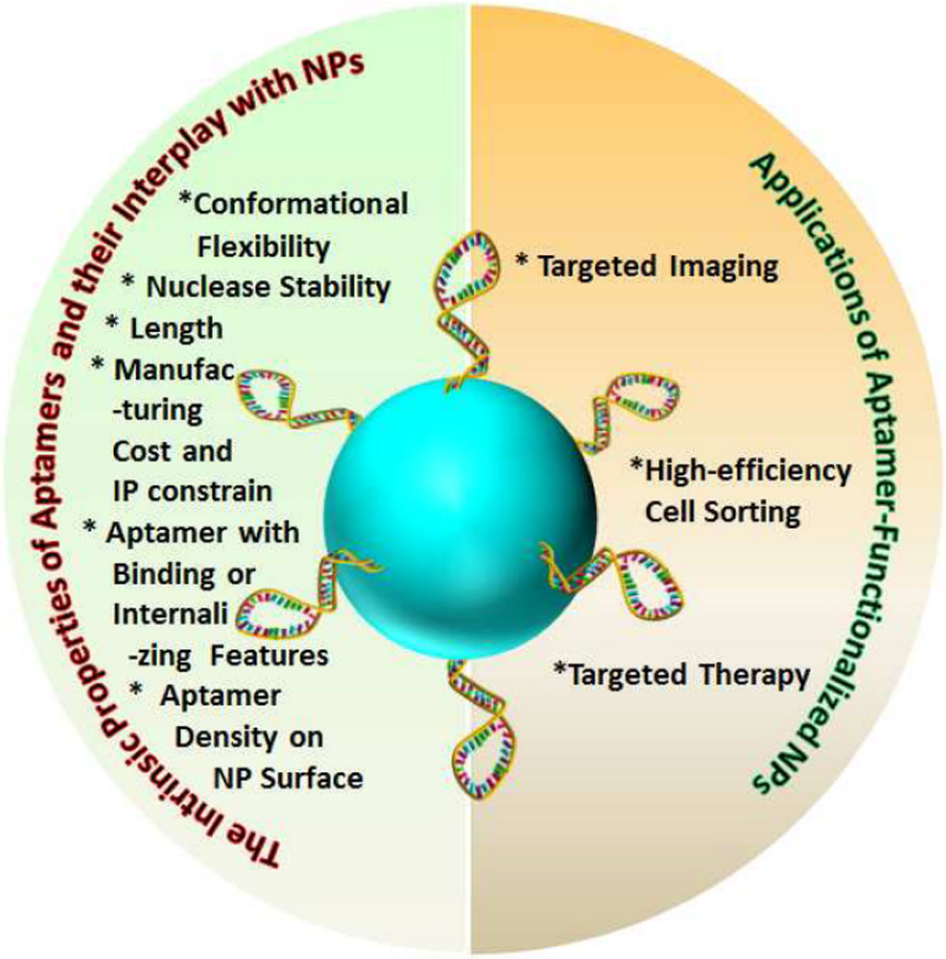


**Figure 2.**

Aptamer-functionalized NPs have been designed for biomedical applications. During the translation of current proof-of-concept designs into *in vivo* applications, the intrinsic properties of aptamers, along with their interplay with NPs, provide some potential challenges and opportunities.

**TABLE 1**

Summary of Aptamer-Functionalized NPs for Medical Applications.

| target molecule | aptamer | selection technique | NP platform | application | reference |
|---|---|---|---|---|---|
| epidermal growth factor receptor (EGFR) | RNA | SELEX against purified proteins | gold NPs | targeted therapy, *in vitro* | 27 |
| prostate-specific membrane antigen (PSMA) | RNA | SELEX against purified proteins | gold NPs | targeted imaging and therapy, *in vitro* | 28 |
| | | | superparamagnetic iron oxide NPs (TCL-SPIONs) | targeted imaging and therapy, *in vivo* | 29 |
| | | | polymeric NPs | targeted therapy, *in vitro* and *in vivo* | 12, 30, 31 |
| mucin 1 (MUC1) | DNA | SELEX against purified proteins | polymeric NPs | targeted therapy, *in vitro* | 32, 33 |
| | | | DNA icosahedral NPs | targeted therapy, *in vitro* | 34 |
| | | | quantum dots | targeted imaging, *in vitro* | 35 |
| adenosine triphosphate (ATP) | DNA | SELEX against small molecules | mesoporous silica NPs | targeted therapy | 36 |
| immunoglobin heavy mu chain (IGHM) | DNA | Cell-SELEX | micelle NPs | targeted imaging and therapy, *in vitro* | 24 |
| | | | supramolecular reticular DNA-quantum dots | targeted imaging, *in vitro* | 37 |
| | | | gold NPs | targeted imaging, *in vitro* | 38, 39 |
| protein tyrosine kinase-7 (PTK7) | DNA | Cell-SELEX | Au-Ag nanorodes | targeted imaging and therapy, *in vitro* | 25, 40 |
| | | | magnetic NPs | cell sorting, *in vitro* | 41 |
| | | | porous hollow magnetite NPs | targeted imaging and therapy, *in vitro* | 42 |
| | | | silica NPs | targeted therapy, *in vitro* | 43 |
| | | | SWNTs | targeted therapy, *in vitro* | 15 |
| not identified | An array of DNA aptamers | Cell-SELEX | magnetic NPs | targeted imaging, *in vitro* | 13 |
| epithermal growth factor receptor 2 (HER2) | RNA | Cell-SELEX | magnetic core–gold shell NPs | cell sorting, targeted imaging and therapy, *in vitro* | 44 |
| | | | gold nano popcorn-attached SWNT | targeted imaging and therapy, *in vitro* | 45 |
| | | | oval-shaped gold NPs | targeted imaging, *in vitro* | 46 |
| not identified | RNA | cell-uptake selection | polymeric NPs | targeted therapy, *in vitro* | 7 |
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| | | | liposomes | targeted therapy, *in vitro* | 14 |
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*ACS Nano*. Author manuscript; available in PMC 2013 May 22.