directed particle aggregation[6,7]. In addition, changes in the LSPR of gold and silver nanostructures have recently been used to observe the binding of biomolecules to their surfaces[8,9]. The sensitivity of the LSPR is such that small fluctuations in the dielectric of the surroundings of the nanoparticle result in significant changes in the optical properties. The magnitude of the shift increases when particles approach one another.

In the present paper, Sönnichsen et al. take advantage of the intense LSPRs of gold and silver nanoparticles and their sensitive dependence on local environment to monitor single nanoparticles and, indirectly, single molecules. The authors begin by observing the scattered light signal from single nanoparticles functionalized with streptavidin when surface-immobilized and illuminated in dark field. Biomolecular interactions are subsequently used to bring two particles near to one another, thereby initiating surface plasmon coupling: gold and silver nanoparticles functionalized with a 33-base DNA sequence terminating in biotin are bound to immobilized streptavidin-coated nanoparticles. Coupling of the surface plasmons leads to a red-shift in the observed light-scattering signal.

To demonstrate that the coupling of the LSPR of a single nanoparticle can be used as a molecular ruler, the authors use Debye screening effects and DNA hybridization interactions to manipulate the interparticle distance. Of particular interest, when the salt concentration is changed, or a target sequence is added, the authors are able to measure a change in the wavelength of scattered light that corresponds to the separation distance of the two particles. A decrease in the salt concentration leads to an increased charge repulsion and a blue-shift in the wavelength of scattered light. Addition of a complementary 33-nucleotide DNA sequence pushes the particles apart owing to an increased duplex 'stiffness' compared with the 33-base single-stranded tether (**Fig. 1**). Importantly, the resulting spectral blue-shift corresponds to the theoretical particle separation induced by DNA duplex formation and supports the notion that plasmon coupling can be effectively used as a molecular ruler. Further, during the hybridization process, a series of 'jumps' in the blue-shifted signal are observed as the complementary DNA sequence begins to react with the single-stranded complement between the two nanoparticles. This behavior provides evidence that a single molecule is responsible for the observed behavior.

The study is important for several reasons. First, it builds upon recent work that demonstrates the exquisite sensitivity of LSPR sensors, which allows important biomolecular interac-

tions to be interrogated on the scale of single molecules[8,9]. Second, the plasmon ruler measures distance fluctuations with nanometer resolution and is potentially accurate over a much longer distance than is FRET (up to 2.5 times the particle diameter)[1]. Third, metal nanostructures give a consistent output signal, potentially superior to that of molecular fluorophores, and are resistant to photobleaching, enabling even single molecules to be stably monitored over extended time periods. This is an important advantage over FRET-based experiments, in which the consistency and lifetime of a good measurement can be unreasonably short.

The technology also faces several challenges to realize its full potential. Molecular fluorophores have the attribute of being small molecules in the size regime of the building blocks of biological macromolecules (amino and nucleic acids). As such, they can be directly incorporated into specific regions of target molecules (e.g., via site-specific unnatural amino acid mutagenesis for proteins[10], PCR-based techniques for nucleic acids or directly during synthesis). In the case of nanoparticle plasmon rulers, the authors report the use of comparatively large gold and silver structures between 20 and 40 nm in diameter. Modifying specific sites in a macromolecule with such nanostructures could prove difficult, depending upon the size of the macromolecule. However, nanoparticle tags of the type described by Sönnichsen et al. could potentially be used to probe interactions between macromolecules (e.g., protein-protein, DNA-DNA or DNA-protein), because both are in the same size regime. Nanostructures could

also be tethered to small molecules, allowing additional possibilities.

Another challenge facing this technology is attaching biomolecules to the surface of nanostructures with a high degree of confidence about the architecture of the resulting structure. The interactions between nanoparticles and DNA and proteins can be quite strong and complex, leading to uncertainties regarding the starting architecture. This issue will have to be addressed on a case-by-case basis to identify compatible biomolecules of interest.

Overall, the authors report a novel method for the interrogation of distance-dependent biomolecular interactions at the single-molecule level. This work builds upon a growing body of literature citing the usefulness of the extreme sensitivity of the LSPR of noble metal nanoparticles to changes in their local environment and could become a very useful addition to the nanotechnology tool kit.

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Receptor-targeted siRNAs

John J Rossi

Protamine-antibody fragment fusions enable delivery of siRNAs to specific cells in mice.

One of the major challenges in transforming small interfering RNA duplexes (siRNAs) from laboratory reagents to therapeutics is developing an effective drug delivery mechanism. Previous efforts to deliver naked

John J. Rossi is at the Division of Molecular Biology, Graduate School of Biological Sciences, Beckman Research Institute of the City of Hope, 1450 E. Duarte Road, Duarte, California 91010, USA.
e-mail: jrossi@bricoh.edu

siRNAs involved high pressure tail-vein injections, resulting in target-specific degradation of a reporter gene transcript in the liver[1]. However, this harsh treatment requires the rapid injection of solutions two-and-a-half times the blood volume of the animal. A considerable improvement in siRNA delivery has been achieved by covalently attaching cholesterol to one of the siRNA strands, which allows direct intravenous, low-pressure injection of the material into mice[2]. In this issue, Song et al.[3] now take *in vivo* delivery one step further by developing

heavy-chain antibody fragment (Fab) fusions that deliver noncovalently bound siRNAs via surface receptors to cells.

RNA interference (RNAi) is a powerful cellular mechanism for targeted downregulation of gene expression. One form of RNAi involves sequence-specific degradation of mRNAs triggered by siRNAs 21–23 nucleotides in length, which are normally produced in cells from longer double-stranded RNA precursors by the ribonuclease III family member Dicer[4]. Two studies in 2001 demonstrated that this Dicer step can be bypassed in mammalian cells *in vitro* when cationic lipids are used as a delivery vehicle for synthetic siRNAs[5,6].

Although the delivery of siRNA to cultured cells is satisfactory for most *in vitro* applications, therapeutic delivery *in vivo* presents an altogether more daunting challenge. Ideally, a delivery mechanism would be capable of binding siRNAs in a reversible manner (to ensure subsequent efficient release of the siRNAs in target cells), allow protection (from nucleases) during transit through the circulation and on release into endosomes, be biocompatible (nontoxic and nonimmunogenic) and biodegradable, and avoid rapid capture and clearance by the liver and kidney.

Some early steps in addressing these challenges have already been made. In a recent study, a significant improvement in siRNA delivery *in vivo* was achieved by covalent attachment of cholesterol to one of the siRNA strands, which were then introduced intravenously, via low-pressure injection, into mice[2]. These studies resulted in effective reduction of the apolipoprotein B and consequently, cholesterol levels in the animals in both liver and jejunum[2].

In the present study, Song *et al.* now tackle the problem of targeting delivery of siRNAs to specific cell types *in vivo*. Their work builds on previous studies showing receptor-mediated uptake of plasmid DNA via association of the nucleic acid with a fusion protein comprising the abundant and highly basic cellular protein protamine and an Fab targeting ErbB2, a member of the epidermal growth factor receptor (EGFR) family[7] (**Fig. 1**). Since protamine is a net positively charged protein, it interacts readily with net negatively charged nucleic acids. Either the entire 51 amino acid protamine peptide or a fragment peptide can be covalently attached to other proteins, such as Fabs.

To test this approach for siRNA delivery, Song *et al.* used a potent, anti-HIV envelope Fab to create a protamine-Fab fusion that binds extracellulary displayed HIV-1 enve-

Figure 1 Proposed receptor mediated endocytosis of antibody fragment-protamine-siRNA complex. The antibody binds to the receptor expressed on the cell surface. The bound complex is endocytosed in clathrin coated pits. The vesicles are uncoated and the siRNA is released from the endosome to enter the RNA induced silencing complex (RISC).

lope glycoprotein. They first asked whether the protamine-Fab conjugate could bind siRNAs and target HIV-1 envelope–expressing cells in culture by simply mixing the conjugate with siRNAs and incubating the complex with cultured cells expressing HIV-1 envelope glycoprotein. Their first set of experiments used fluorescently labeled siRNAs to monitor the delivery method and the experiment worked like a charm. Only cells that expressed the HIV-1 envelope took up the fluorescently tagged siRNAs.
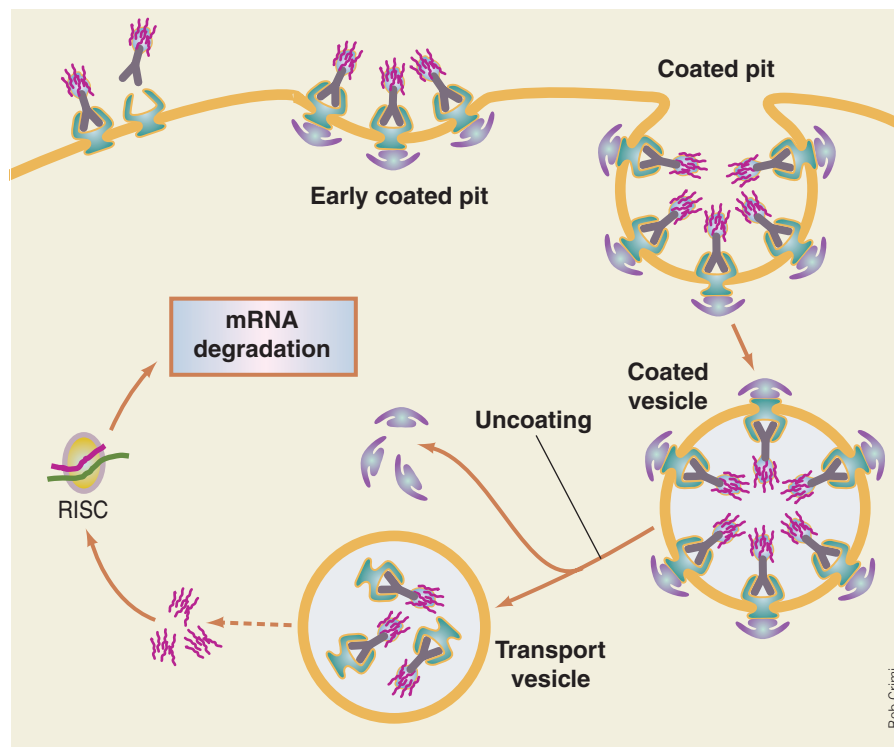
Song *et al.* next asked if siRNAs delivered by this method were functionally active against enhanced green fluorescent protein (EGFP) in envelope-expressing cells. These experiments resulted in sequence-specific knockdown of EGFP only in cells expressing HIV-1 envelope and only with anti-EGFP siRNAs. The authors then demonstrated that an anti-HIV *gag* siRNA could be delivered to cultured T-lymphocytes infected with HIV-1. Again, they observed specific targeting to the envelope-expressing cells, with significant inhibition of HIV p24 gag antigen only with the anti-*gag* siRNA:protamine–Fab complex. The siRNA reduced the levels of secreted viral gag antigen by over 70%. Significantly, this experimental result overcomes the difficulties associated

with transfecting T-lymphocytes and opens the possibility of using this approach in the treatment of HIV infection.

Receptor-mediated delivery of siRNAs in cell culture is clearly useful, but the critical test is whether or not such a delivery scheme can work *in vivo*. To explore this, Song *et al.* engineered a melanoma cell line that expressed the HIV-1 envelope glycoprotein and injected these cells into the flanks of mice. For targeting controls, they injected unmodified (non-envelope expressing) melanoma cells into a different group of mice. A cocktail of siRNAs targeting *c-myc*, *MDM2* and vascular endothelial growth factor mRNAs were mixed with the protamine-Fab conjugate and injected intravenously or intratumorally either on the same day or 1 or 3 days after the tumor-forming cells were implanted. The results were consistent with those obtained in cell culture; only tumors expressing the HIV-1 envelope and treated with the siRNA:protamine–Fab complex showed reduced tumor volume. No inhibition of tumor growth was observed when naked siRNAs were injected intratumorally. It is important to note that intravenous delivery worked in this model, but was not as effective as the intratumoral injection. The

reason for this is not clear and certainly merits further investigation.

To verify that the delivery system was not confined to the HIV-1 envelope system, the authors demonstrated that an anti-ErbB2 Fab fused to a protamine fragment could selectively deliver siRNAs to a breast cancer cell line expressing the ErbB2 growth factor receptor. In these experiments, they demonstrated receptor-mediated delivery of siRNAs targeting Ku70 and observed effective knockdown of this target only in cells expressing the ErbB$_2$ receptor.

One specific safety concern associated with siRNA molecules is their potential to activate the interferon (IFN) pathway. Recent reports have demonstrated that certain motifs in siRNAs are potent stimulators of IFN-α and β via interactions with toll-like receptors (TLRs) 3 and 7 (refs. 8–10). Song et al. assayed for IFN pathway gene induction in cell culture and saw no specific stimulation by the siRNA:protamine–Fab complexes; nor did they observe any toxicity in the in vivo analyses. Previous studies[9,10] revealed that the primary IFN-responding cell types are plasmacytoid dendritic cells, which express TLRs 3 and 7. Thus, this technique for selective receptor targeting of siRNAs to cells in vivo may prove an important approach to mitigate the IFN response in future siRNA therapies.

Where do we go from here with this technology? The use of humanized, monoclonal antibodies for the treatment of a variety of diseases is becoming an increasingly important therapeutic modality. Song et al.'s study offers the possibility of using Fabs to ferry siRNAs to tumor cells and other therapeutically important targets. The hope is that the combined use of therapeutic antibodies and siRNAs will provide synergy in the treatment of diseases such as cancer. Given the excitement over the use of receptor-targeting monoclonal antibodies and the power of RNAi in downregulating mRNAs required for cell proliferation, the combination of the two could transform the treatment of many diseases. There are also many ligands other than antibodies that could be recruited for receptor-mediated delivery using the protamine-fusion technology.

Certain technological hurdles must be overcome, however, before the siRNA:protamine–fusion delivery technology can be more widely adopted. These relate to the agent used to target the siRNA:protamine complex and to the complex itself. In the former case, the use of large antibody fragments or protein ligands in the protamine fusion could potentially alter siRNA targeting specificity. In the latter case, the amount of siRNA bound to protamine may limit efficacy. In the Song et al. study, for example, the relative concentrations of siRNAs required to effect targeted silencing were relatively high due to limitations in the number of siRNA duplexes that are complexed with the protamine fusion protein (approximately six per complex). This might be addressed by introducing selective backbone modifications into the siRNAs to enhance the number of protamine-bound molecules. At the same time, modifications must neither compromise binding affinity for target mRNA sequence nor prevent the siRNA from releasing efficiently from the protamine complex in the endosome.

Other modifications to the siRNAs backbone might be envisioned to enhance resistance to degradation in the harsh endocytic environment of cells or to reduce siRNA clearance by hepatocytes in the liver and kidney. As always, judicious choice of target regions within an mRNA sequence will likely be paramount in ensuring optimal therapeutic potency.

With these technical issues in mind, the Song et al. methodology for targeted siRNA delivery in vivo provides a basis for further studies. The true test of this innovative delivery method will be how well it can be reproduced with other targeting proteins. The relative ease of making these complexes should lead to the widespread testing of this receptor-mediated delivery approach in the months to come.

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Viral-mediated plant transformation gets a boost

Stanton B Gelvin

Making the tobacco mosaic virus RNA genome more 'mRNA-like' enhances its ability to direct the synthesis of recombinant proteins in plants.

Viral-based transient expression systems[1,2] for plants have certain advantages over stable transformation systems that employ Agrobacterium tumefaciens or particle bombardment. These include the rapidity with which one can go from vector construction to plant expression and the ability of certain viruses to replicate to high levels and travel systemically throughout the plant, thereby rapidly turning the plant into a 'factory' for the production of particular gene products. Despite these advantages, transient expression systems traditionally have been limited by the low infectivity of viral vectors carrying average-sized or large transgenes. In this issue, Marillonnet et al.[3] describe modifications to tobacco mosaic virus (TMV) expression vectors that permit efficient infection and viral replication in almost 100% of plant leaf cells. The result is an expression system capable of a massive increase in the amount of recombinant protein made in planta.

Over the past 25 years, two approaches have been developed for the high-level expression of foreign proteins in higher plants: the first involves the stable incorporation of transgenes (generally using A. tumefaciens–mediated transformation or particle bombardment) into either the nuclear or the chloroplast[4] genome; the second involves transient expression of transgenes, frequently from vectors derived from TMV[5] or potato virus X (PVX)[6]. Although transient expression systems are often preferred in experimental settings due to their speed and ease of application, they also encounter problems, such as silencing (which can be counteracted by using viral silencing suppressors)[7] and containment (which can be mitigated using mutant viruses that cannot produce capsid proteins)[2].

When inducing transient transgenesis, plants can be inoculated with recombinant viral RNA

Stanton B. Gelvin is in the Department of Biological Sciences, Purdue University, 915 W. State St., West Lafayette, Indiana 47907-1392, USA.
e-mail: gelvin@bilbo.bio.purdue.edu