Development of Nanoparticle Libraries for Biosensing

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Magnetic and magnetofluorescent nanoparticles have become important materials for biological applications especially for sensing, separation, and imaging. To achieve target specificity, these nanomaterials are often covalently modified with binding proteins such as antibodies or proteins. Here we report on the creation of nanoparticle libraries that achieve specificity through multivalent modification with small molecules. We explore different synthetic routes to attach small molecules with anhydride, amine, hydroxyl carboxyl, thiol, and epoxy handles. We show that the derived nanomaterials have unique biological functions, possess different behaviors in cell screens, and can be used as substrates for biological screens.

INTRODUCTION

To use emerging nanomaterials more efficiently in biological applications, it is often essential to functionalize them with organic ligands to impart precise biological functions (*1*−*3*). Conversely, the creation of entire libraries of nanomaterials could greatly accelerate screening for use in biomedical applications. Magnetic and magnetofluorescent nanoparticles (MNPs and MFNPs) have become important materials in biomedicine for sensing (*4*), magnetic separation, and/or in vivo imaging (*5*). While prior research has focused on the design of specific nanoparticles (*6*, *7*, *20*) or polymers (*9*), detailed procedures for the synthesis and evaluation of surface-modified nanoparticle libraries using different conjugation strategies has not been described. The goal of this study was to develop, optimize, and characterize parallel conjugation chemistries for the creation of magnetofluorescent nanoparticle libraries.

We describe methods of attaching different types of small molecules with reactive handles (anhydride, amino, hydroxyl, carboxyl, thiol, and epoxy) to an aminated magnetofluorescent nanoparticle (amino-CLIO-FITC (*9*)). Although a variety of antibodies and proteins have been conjugated to this nanoparticle, small molecules of nonbiological origin were employed to avoid cost and regulatory issues if a member of the library is subsequently selected for scale-up and clinical use. Small molecules (<2 kDa) can also be separated from the nanoparticle (∼1000 kDa) easily. For each of the chemistries, a method of ensuring that modified particles have been produced is provided. This is particularly important when large numbers of surface-functionalized nanoparticles are made and screened for activity, since an unsuccessful nanoparticle modification cannot be readily distinguished from successful nanoparticle modification with inactivity due to a lack of recognition by a biomolecule. The presence of these types of errors in a database can thus lead to erroneous conclusions regarding structure activity relationships and the misdesign of subsequent libraries. We also show how modified nanoparticles can be used for library screens, analyte sensing, and microarray printing.

EXPERIMENTAL PROCEDURES

Materials. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), sulfosuccinimidyl ester (sulfo-NHS), and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Pierce. *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and succinimidyl iodo acetate (SIA) were purchased from Molecular Biosciences. All other chemicals were purchased from Sigma Aldrich and used as received.

Magnetic Nanoparticle. The superparamagnetic nanoparticle used in this study consisted of an iron oxide core (5 nm diameter, 2064 Fe per average nanoparticle) with a thick shell of dextran (*10*). Amino-CLIO nanoparticles were synthesized by cross-linking the dextran coating with epichlorohydrin and reacting it with ammonia to provide primary amine groups (*11*). The number of amines per nanoparticle was determined using the SPDP method described previously (*12*). To attach FITC to amino-CLIO, a 2.0 mL DMSO fluorescein isothiocyanate (FITC, 5.0 mg, 12.8 mol) solution, 10 mL of amino-CLIO (10 mg/mL Fe) in 0.01 M sodium citrate buffer, pH 8.0 was added. Subsequently 100 μL of *N*,*N*-diisopropylethylamine (0.57 mmol) was added as catalyst. The mixture was incubated at room temperature overnight, and unreacted FITC was separated by gel filtration (Sephadex G-25), equilibrated, and eluted with PBS buffer, pH 7.4 (Cambrex, Rockland, ME). The number of FITC conjugated to CLIO was determined spectrophotometrically (extinction coefficient of 73 000 M^{-1} cm^{-1} at 494 nm). All batch syntheses were characterized by determining size by laser light scattering (38 nm), magnetic relaxivities (R1 of 21 mM s^{-1}, an R2 of 62 mM s^{-1}), magnetic susceptibility, and number of amines (n = 62 per 2000 Fe) and FITC (n = 2) conjugated.

Surface Modification of Nanoparticles. *Modification by Anhydrides.* To a 100 μL anhydride (50 mM, 5 μmol) DMSO solution, 200 μL of amino-CLIO (5.0 mgFe /mL, 0.4 μmol of amine) in 0.1 M bicarbonate buffer, pH 8.5 was added. The mixture was incubated at room temperature for 2 h with gentle shaking. Unreacted anhydride was separated with Sephadex G-25 column and eluted with PBS buffer, pH 7.4.

Modification by Amine Groups. We first converted amine groups on amino-CLIO to carboxyl groups by reaction with succinic anhydride at the conditions described above for the anhydride/CLIO surface modification. After purification of the sample, carboxylated CLIO was stored in MES buffer (50 mM MES, 0.1 M NaCl), pH 6.0 at a concentration of 5.0 mg/mL Fe. To react with amine molecules, a 200 μL COOH-CLIO solution was first reacted with EDC (0.96 mg, 5 μmol) and sulfo-NHS (1.1 mg, 5 μmol) at room temperature for 60 min. The mixture was then purified through Sephadex G-25 column eluted with PBS buffer, pH 7.4. Subsequently 100 μL of amine

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compound in DMSO (50 mM) was added to the above CLIO solution and reacted for 2 h at room temperature. Unreacted amines were separated by a Sephadex G-25 column eluted with PBS buffer, pH 7.4.

Modification by Hydroxyl Groups. Amino groups on the amino-CLIO nanoparticle were first converted to hydroxyl groups by reaction with SIA. Two milligrams of amino-CLIO was reacted with SIA (1.0 mg, 3.5 μmol) in PBS buffer, pH 7.4. The mixture was incubated at room temperature for overnight to allow displacement of iodine by hydroxyl groups. Hydroxylated CLIO was purified by a Sephadex 25 column eluted with PBS buffer, pH 7.4. Purified OH-CLIO was subsequently precipitated and washed with cold acetone for several times. One milligram of precipitated OH-CLIO was resuspended in 200 μL of anhydrous DMSO and activated by $SOCl_2$ (5 μL, 68.5 μmol). After 5 min reaction at room temperature, a drop of H_2O was added to decompose the excess $SOCl_2$. Subsequently, 200 μL of alcohol (50 mM) in DMSO was added with *N*,*N*-diisopropylethylamine (10 μL, 50 μmol) as catalyst. The mixture was incubated at room temperature for 3 h, and hydroxylated CLIO conjugates were purified by a Sephadex G-25 column eluted with PBS buffer, pH 7.4.

Modification by Carboxyl Groups. Amino-CLIO in PBS buffer, pH 7.4 was first exchanged with MES buffer (50 mM MES hydrate, 0.1 M NaCl), pH 6.0, and the solution was concentrated to 5.0 mg/mL. 100 μL (50 mM) of carboxylic compound in DMSO was added to 200 μL of amino-CLIO (5.0 mg/mL Fe) in MES solution, pH 6.0. This was followed by the addition of excess EDC (0.96 mg, 5 μmol) and sulfo-NHS (1.1 mg, 5 μmol) in 100 μL of DMSO. The reaction proceeded at room temperature for 2 h, and the product was purified by a Sephadex G-25 column and eluted with PBS buffer, pH 7.4.

Modification by Thiols. Amino-CLIO was first reacted with SPDP.[7] After purification of the sample, 200 μL of SPDP derived CLIO (5.0 mg/mL Fe) in PBS buffer, pH 7.4 was mixed with 100 μL of thiol compounds (50 mM) in DMSO. The reaction proceeded for 2 h at room temperature. The CLIO conjugate was separated by a Sephadex G-25 column and eluted with PBS buffer, pH 7.4. For the MRS assays, thiolated influenza hemagglutinin (HA) peptide with a cysteine terminus (YPYDVPDVAGGC) was synthesized by using Fmoc chemistry on Rink amide resin (Calbiochem, NovaBiochem) and was purified by reverse phase HPLC. The molecular weight of HA was confirmed by MALDI-TOF.

Modification by Epoxide Groups. Amino-CLIO in PBS buffer, pH 7.4 was first exchanged to a 0.15 M sodium bicarbonate buffer, pH 8.5, and the solution was concentrated to 5.0 mg/mL. To 200 μL above solution, 100 μL (50 mM) of epoxide compound in DMSO was added. The reaction proceeded at 80 °C for 4 h, and the product was purified by a Sephadex G-25 column and eluted with PBS buffer, pH 7.4.

Surface Characterization. *Surface Modification by Amine Loss.* The loss of amines on anhydrides or carboxyl reacted amino-CLIO was determined using with SPDP as reagent (*12*). SPDP is preferred as a reagent for amine group analysis over more conventional amine assay methods because after treatment with dithiothreitol the pyridine-2-thione (P2T) can be separated from iron based on size. Iron absorbs strongly in the ultraviolet region, a feature that interferes with many amine determining reagents. After reaction with SPDP, the nanoparticle is treated with a reducing agent (TCEP) and the released P2T separated from the nanoparticle by a spin column. P2T absorption can now be determined without the strong nanoparticle absorption at the wavelength of the P2T (343 nm). In a typical experiment, 150 μL of 50 mM SPDP solution in DMSO was added to 50 μL (2 mg Fe) of CLIO in PBS, pH 7.4. The mixture was allowed to react at room temperature for 1 h and purified by Sephadex 25 column. This solution was subsequently added with 50 μL of TCEP (Pierce) and UV-Vis absorption was read at 343 nm. The number of amines was calculated based on an extinction coefficient of 8080 M^{-1} cm^{-1} for P2T.

Magnetic Relaxation Switch Assay. The bioactivity of surface-modified CLIO nanoparticles was examined by their interaction with their corresponding binding molecules, a process that can be monitored by the change in the proton spin−spin relaxation time (T2) (*4*). When nanoparticles bind to their corresponding binding proteins, nanoscale aggregation occurs and the T2 decreases (Figure 2). Control samples were prepared with unmodified nanoparticles. Surface-modified nanoparticles at 10 μg/mL iron in PBS buffer, pH 7.4 were mixed with their binding protein at concentrations, which induced maximal T2 changes determined on a minispec MR spectrometer. Imaging was performed after a 30 min incubation of surface-modified nanoparticles with their corresponding binding proteins at 37 °C. T2 was measured by NMR imaging acquired in a 49 well plate using a 4.7 T superconducting magnet (Brucker, Karlsruhe, Germany). Imaging sequences included multi-echo T2-weighted spin−echo sequences with echo times (TE) ranging from 20−160 ms in increments of 20 ms and a repetition time (TR) of 2000 ms.

Array Printing and Imaging. Two strategies were used to print modified nanoparticles to glass slides. The first method utilized epoxy-modified glass slides. In this method, CLIO compounds were dissolved in 0.1 M carbonate buffer, pH 9.6 at a concentration of 0.1 mg/mL Fe. Each compound was spotted on epoxy glass slides in quadruplicate using a 0.5 μL pipet. Spotted slides were incubated at 80 °C in a humid chamber for 30 min. The alternative method used carboxylated glass slides, which were first activated with EDC and NHS in MES buffer (50 mM MES, pH 6.0) for 30 min followed by drying. Nanoparticles in 0.1 M bicarbonate buffer, pH 8.5 were spotted in quadruplicate using a 0.5 μL pipet. Spotted slides were allowed to dry under inert gas and stored at 4 °C for further use.

To reveal the binding of corresponding proteins to modified nanoparticles, slides were first washed for 2 h with DMF, and 1 h each with 2-propanol and PBS buffer (pH 7.4, containing 0.1% Tween-20). The slides were then blocked for 1 h by incubation with 0.1 M carbonate buffer containing 3% BSA, pH 8.5. After a brief rinse with PBS, fluorescently labeled target proteins (streptavidin-rhodamine, anti-IgG-Cy5.5) were then added at a concentration of 0.1 mg/mL in PBS supplemented with 1% BSA. The slides were incubated for 30 min and then washed 10 times with PBS. The slides were then dried at ambient temperature and imaged with a fluorescence imager. The following filter sets were employed: 680/700 nm excitation/emission filter set (2 s exposure); 560/590 nm excitation/emission filter set (1 s exposure).

Cell Uptake Experiments. U937 cells were obtained from the American Type Tissue Culture Collection (ATCC) and maintained according to ATTCC protocol. For differentiation into macrophages, the nonadherent monocyte-like undifferentiated U937 cells were induced to differentiate by a 48-h exposure to 40 nM PMA (Sigma). After addition of PMA, cells were plated onto gelatin-coated 96-well tissue culture plates. Cells were incubated with 0.1 mg/mL Fe of the indicated CLIO derivatives for 4 h at 37 °C in the presence of 5% CO_2. Following incubation, wells were washed 3× with PBS/0.1%BSA/0.05%Tween-20 wash buffer to remove any surface-bound materials. Cells were then analyzed via an immunoassay to quantitate FITC concentrations (*13*). All experiments were performed in triplicate. For each screening experiment, the uptake of FITC (pM) was log10 transformed, and the mean was determined for each cell line.

Table 1. Summary of Surface Modification

type	reagents	examples	binder	modification[a]	MRSW[b]
anhydride	direct	1,8-naphthalic anhydride	lysozyme	24	23 ms
amine	EDC, NHS	D-glucosamine	concavalin A	36	69 ms
alcohol	SIA, $SOCl_2$	digoxigenin	MAB to dig	30	65 ms
acid	EDC, NHS	folic acid	MAB to folate	32	63 ms
thiol	SPDP	HA peptide	MAB to HA	34	81 ms
epoxide	direct	glycidoxypropyltrimethoxy silane	glass slide	40	NA

[a] Refers to average number of small molecule bound per nanoparticle as determined analytically and assuming an average core of 2,064 Fe/nanoparticle. [b] T2 were measured in PBS buffer, pH 7.4, 37 °C, 0.47 T at an iron concentration of 10 μg/mL. Data are expressed as change in T2 relaxation times.

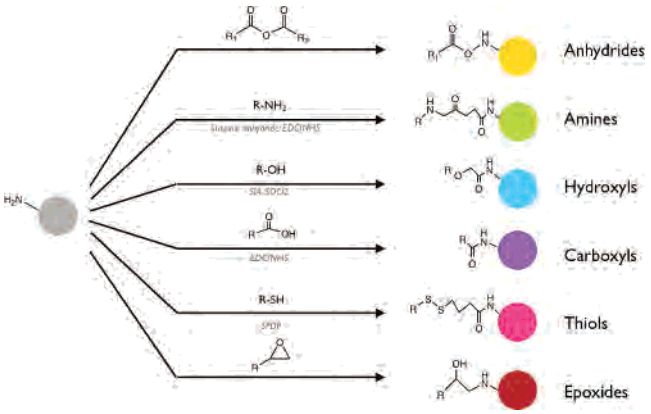

Figure 1. Conjugation strategies. Aminated magnetofluorescent nanoparticles (amino-CLIO-FITC) were reacted with small molecules with different reactivities. Reactions were analyzed by determining loss of amines[12] and the magnetic relaxation switch assay (see Figure 2).

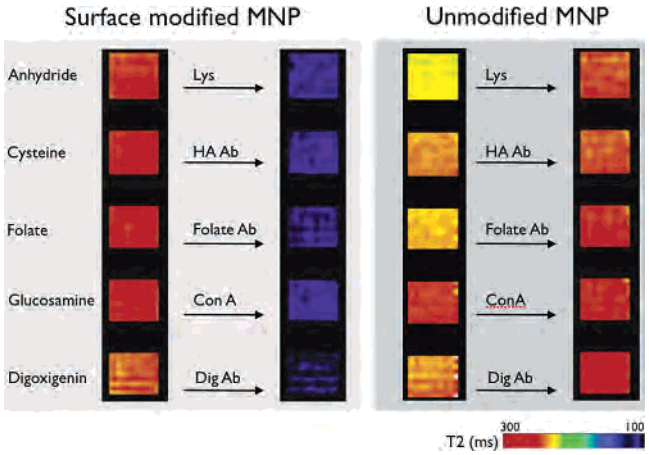

Figure 2. MRS assay confirms bioactivity of derivatized nanoparticles. Nanoparticle preparations modified according to Table 1 (left) or unmodified (right) were incubated with their corresponding binding protein that induces nanoparticle clustering and thus T2 decreases. It is clearly shown that T2 decreases dramatically when modified nanoparticles react with corresponding binding proteins (second column), while nonmodified nanoparticles did not show any visible binding interactions (third and fourth columns).

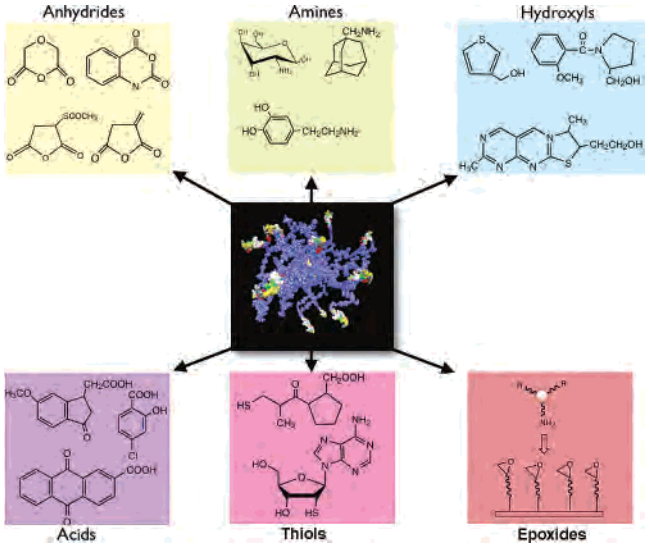

Figure 3. Nanoparticle library. Examples of small molecules attached to amino-CLIO to create a 96-member library (to fill one microtiter plate) that was subsequently used (see Figures 4 and 5).

RESULTS AND DISCUSSION

The first step toward creation of libraries was to optimize the conjugation of six types of small molecules with "reactive handles" to a magnetofluorescent nanoparticle: anhydrides, amines, hydroxyls, carboxyls, thiols, and epoxides (Figure 1). The starting material consisted of a cross-linked dextran coated aminated magnetic nanoparticle of 38-nm diameter that had been reacted with two molecules of FITC (*9*). Each nanoparticle contained 62 amines and a magnetic core containing 2064 Fe per nanoparticle (*14*). For the specific task at hand, we chose representative examples of each class of small molecules to optimize conjugation protocols. The representative examples included diglycolic anhydride, glucosamine, digoxigenin, folic acid, cystein-containing HA peptide, and glycidoxypropyltrimethoxy silane. These specific molecules were chosen because each has a corresponding binding protein, which can be used to examine the bioactivity of the surface-functionalized nanoparticle.

The conjugation of anhydride molecules to amino CLIO was performed in bicarbonate buffer at pH 8.6. Higher pH facilitates the reaction of anhydride group and amino group on CLIO. There were an average of 24 modified amines per nanoparticle. To efficiently attach amine groups to amino-CLIO, we first converted primary amines on the nanoparticle to carboxylic groups. Subsequently, EDC and sulfo-NHS were used to conjugate carboxylate CLIO to amine compounds. To link hydroxyl groups to CLIO, we first treated amino CLIO with succimidyl iodoacetate (SIA). Hydrolysis of SIA-treated CLIO resulted in the conversion of amine groups on CLIO to hydroxyl groups. Hydroxylated CLIO was first precipitated by cold acetone and then resuspended in anhydrous DMSO. Subsequently, $SOCl_2$ was added to activate hydroxyl groups. Excess $SOCl_2$ was decomposed by a small amount of water. The alcohol molecules were added to chlorinated CLIO with the addition of a small quantity of NaOH as catalyst. With respect to carboxyl conjugation, we compared several conjugation methods and found that EDC conjugation was the most efficient. In this reaction, the carboxylic molecule (in DMSO) was first activated by EDC in MES buffer, and the reaction to amino-CLIO was accomplished in room temperature in 3 h. Unreacted molecules were separated by a PD-10 column eluted with PBS buffer, pH 7.4. For the reaction of thiol molecules, CLIO was first activated with SPDP, and subsequently thiol molecules were conjugated. Attachment to epoxides was performed in 0.15 M sodium bicarbonate buffer, pH 8.5 by adding epoxides in DMSO. Reaction proceeded for 4 h, and the product was purified by Sephadex G-25 column chromatography.

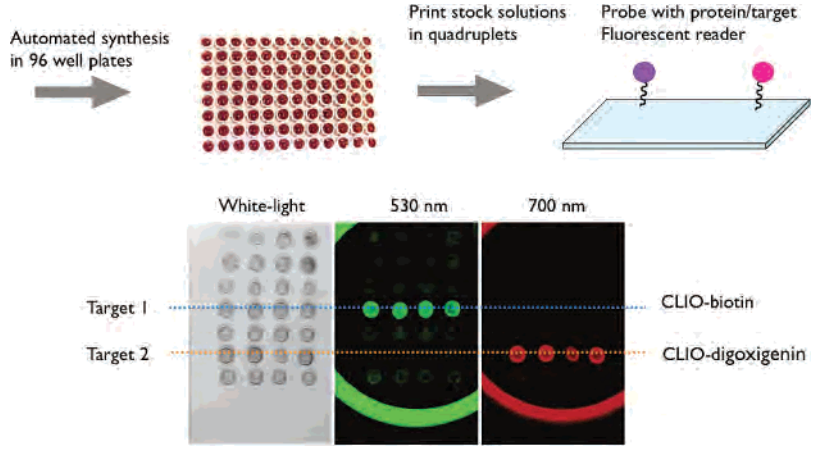

Figure 4. Microarray feasibility experiments. Surface-functionalized nanoparticles of different types were printed onto epoxy glass slides and then washed. Fourth row from top: CLIO-biotin; sixth row from top: CLIO-digoxigenin; remaining rows contained unmodified nanoparticles. Slides were incubated with a mixture of the binding proteins, streptavidin-rhodamine, and anti-digoxigenin, revealed by anti-IgG-Cy5.5, and scanned using a fluorescent reader. Note that the targets are clearly separated in the different channels. All experiments were performed in quadruplicate.

Surface modification of nanoparticles was monitored by two different assays, specifically developed for MFNP: (a) an assay for the modification of amines (amine loss) using SPDP as a reagent (*12*) and (b) a magnetic relaxation switch assay to demonstrate that both surface modification and the activity of the surface with a biological molecule (*4*). The strong absorption from iron oxide between 250 and 450 nm, nanoparticle superparamagnetism, and the presence of both the oxidizing ferrous ion and reducing ferric ions, mean that the nanoparticle interferes with many analytical methods that work well with other materials. The use of SPDP for amine determination permits separation of P2T, released from the nanoparticle with a reducing agent, from iron before quantitation. Second, biomolecules when attached to surfaces can interact with the surface and lose bioactivity (surface denaturation). On average, small molecule modification resulted in the attachment of 20−40 functional groups per nanoparticle. Table 1 summarizes the overall chemical conjugation and characterization conditions. The magnetic relaxation switch assay (MRS) was used to verify the bioactivity of a surface-functionalized CLIO made with each of the conjugation chemistries. This assay is based on the fact that water proton spin−spin relaxation time (T2) are altered upon self-assembly when surface-functionalized CLIO nanoparticles react with a biomolecule in solution (*4*, *15*−*17*). As can be seen from Figure 2, each representative example of a surface-modified nanoparticle showed a significant decrease in T2 relaxation time, whereas unmodified nanoparticles did not. Thus, each of the surface modification reactions employed was capable of producing a bioactive surface.

We next set out to develop a larger diverse library set based on the optimized conjugation strategies discussed above. Figure 3 lists some of the representative examples of each of the classes of small molecules bound to the nanoparticle. Overall, we created a nanoparticle library consisting of 96 compounds (Figure 4). This library was used to perform two different sets of biological experiments: (a) microarray screen after compounds were printed on glass slides and (b) fluid-phase experiments to determine nanoparticle uptake into macrophages (Figure 5). We first wished to determine if the nanoparticles could be printed onto glass slides to ultimately allow high-throughput screening against purified target proteins. We experimented with two different conjugation schemes: attachment of nanoparticles to epoxide-coated glass slides and to carboxylated glass slides. Either method resulted in visible attachment of nanoparticles to the glass surface, both by pipet spotting (Figure 4) as well as a by pin-printing. To determine whether functionalized nanoparticles would retain the ability

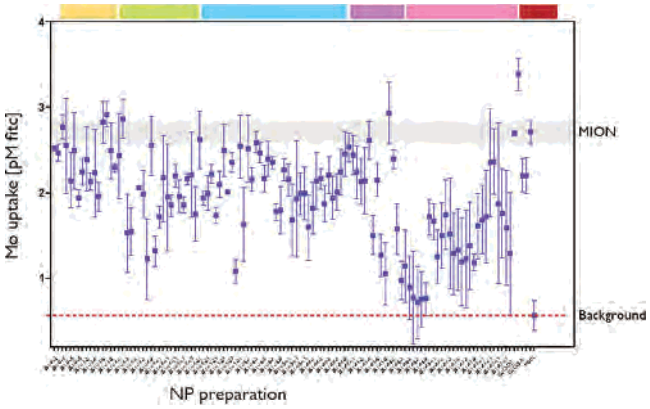

Figure 5. Screening of nanoparticle library in macrophages. Small molecule modified nanoparticle preparations were incubated with U937 macrophages, and cellular uptake was determined by a FITC-mediated immuno ELISA assay.[13] Note the variability in uptake upon small molecule modification compared to base material (MION).

to interact with binding proteins, we tested biotinylated and digoxigenated nanoparticles against fluorescent streptavidin-Cy3 and anti-digoxigenin-Cy5.5, respectively. As is shown in Figure 4, there was good distinction between the signals from the different fluorochromes.

We and others have previously shown that macrophages represent a prime target for systemically injected nanomaterials (*2*, *18*). To develop functionalized nanomaterials targeted to other cell types, it would therefore be desirable to minimize macrophage uptake of the parent compound. We therefore performed a cell-based screen of all synthesized nanoparticle preparations and compared binding/uptake to preparations currently in clinical use (MION-like dextran-coated compounds). As is apparent in Figure 5, several nanoparticle preparations had lower macrophage uptake than starting materials. More in-depth screens in primary human isolated macrophages are currently ongoing to identify novel nanomaterials with limited a priori macrophage affinity.

In conclusion, we have developed a series of highly general methods to modify the surface of magnetofluorescent nanoparticles with small molecules and demonstrated that these nanoparticles achieve specific interactions with protein targets. Analytical methods have been developed to ensure that the desired surface modification of nanoparticles has occurred, and magnetic relaxation switch assays have been used to demonstrate bioactivity, as the reaction of surface-modified nanoparticles with their corresponding binding protein. All materials used for

the modification of nanoparticles were low molecular weight and of nonbiological origin. The current approach has the potential to generate a larger and more diverse library of functional nanomaterials toward numerous biological targets. Moreover, these methods are readily adapted to automated synthesis and suitable for high-throughout screening of the interaction of surface-modified nanoparticles with specific types of cells or molecular targets.

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