Cell-specific targeting of nanoparticles by multivalent attachment of small molecules

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Nanomaterials with precise biological functions have considerable potential for use in biomedical applications. Here we investigate whether multivalent attachment of small molecules can increase specific binding affinity and reveal new biological properties of such nanomaterials. We describe the parallel synthesis of a library comprising 146 nanoparticles decorated with different synthetic small molecules. Using fluorescent magnetic nanoparticles, we rapidly screened the library against different cell lines and discovered a series of nanoparticles with high specificity for endothelial cells, activated human macrophages or pancreatic cancer cells. Hits from the last-mentioned screen were shown to target pancreatic cancer *in vivo*. The method and described materials could facilitate development of functional nanomaterials for applications such as differentiating cell lines, detecting distinct cellular states and targeting specific cell types.

One of the emerging goals of nanotechnology is to functionalize inert and biocompatible materials to impart precise biological functions. Several novel materials have recently been described for diagnostic or therapeutic use¹⁻³, including quantum dots⁴⁻⁶, polymers^{7,8} and magnetofluorescent nanoparticles^{9,10}. Considerable effort has been directed toward rational surface modifications and coatings to modulate pharmacokinetic properties (e.g., blood half-life, elimination and biodegradation), toxicity, immunogenicity and efficient targeting. Targeting has generally been achieved by conjugating nanoparticle surfaces to antibodies. Although this approach has succeeded for in vitro sensing^{11,12}, its in vivo application has proved more challenging because of cost, limited shelf life, regulatory hurdles and potential immunogenicity after repeat injections of such preparations¹³. Another targeting approach with promising initial results involves conjugation of nanoparticles to peptides^{14,15} but synthetic costs can be high.

To take advantage of a more diverse chemical space, we hypothesized that small-molecule modifications could change the biological properties of nanoparticles and thereby permit site-specific targeting through small molecule-mediated multivalent binding to cell-surface receptors. To date, the potential of such small-molecule approaches for the design of nanoparticle surfaces has not been realized, primarily because of a lack of a general method to modify surfaces rapidly, to characterize these modified surfaces chemically and to rapidly screen the resulting nanomaterials for biological activity.

Here we describe the creation of a small-molecule nanoparticle library for the rapid development of magnetofluorescent reporters. We show that a union between nanotechnology and small-molecule chemistry can facilitate development of a wide range of nanomaterials for biomedical applications. Specifically, we screened a model nanoparticle library against different cell lines and states. We identified nanomaterials that discriminate among distinct cell types, or among different physiological states of a given cell type.

RESULTS

Synthesis of nanoparticle library

The first step towards creation of the nanoparticle library was to identify biologically and chemically suitable nanoparticles that could be detected by magnetic and fluorescent means and could be chemically modified. We used magnetofluorescent nanoparticles^{9,10} as starting material because such preparations can be made with high (R2 > 30 mMsec⁻¹) magnetic relaxivity, because related materials are biocompatible and in clinical use¹⁶, and because aminated base materials facilitate conjugation of small molecules through sulfhydryl, carboxyl, amine and anhydride chemistries (**Fig. 1e**).

Using a modified robotic system, we conjugated 146 different small molecules to nanoparticles in array format. Previous feasibility studies had identified several classes of small molecules that combine water solubility, conjugatability, biocompatibility and chemical diversity. In general, we focused on small molecules (MW < 500 Da) with the chemical functional groups of primary amines, alcohols, carboxylic acids, sulfhydryls and anhydrides (**Fig. 1** and **Supplementary Table 1** online), and excluded compounds known to bind proteins. On average, 60 small molecules were attached per 38-nm nanoparticle. All synthesized and purified nanoparticles were water-soluble, magnetic and fluorescent (**Fig. 1d**) and could be stored for prolonged periods. Nanoparticle libraries were stored in multiwell plates until use in cell-based high-throughput screens.

Screening identifies unique nanoparticles

We next tested the nanoparticle library for its effects on mammalian cells. We were particularly interested in whether small-molecule

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surface modification can be used to: (i) change the cellular affinity of nanoparticles, (ii) develop materials that discriminate among closely related functional states of cells, for example, resting and activated (disease-associated) macrophages, (iii) develop disease-specific targeting agents without prior knowledge of a specific target and (iv) develop more efficiently targeted nanomaterials.

Figure 2 summarizes the results of over 30 screening experiments. The heat map represents the log of mean cellular uptake of different nanoparticles in five different cell types. Human umbilical vein endothelial cells (HUVEC), primary resting human macrophages, granulocyte macrophage colony stimulating factor (GM-CSF)-stimulated human macrophages, a U937 human macrophage-like cell line and human pancreatic ductal adenocarcinoma cells were all probed in quadruplicate. Each row represented a different nanoparticle preparation. Cellular uptake varied over three orders of magnitude (red, low; green, high) ranging from 2-2,239 fg Fe/cell. Notably, there was substantial diversity in cellular uptake among the different nanoparticle compounds, both within a given cell type and among different cell types. For example, for the human pancreatic cancer cell line PaCa-2, uptake varied

from 3-1,065 fg Fe/cell among the different preparations $(1.1 \times 10^4 - 5.5 \times 10^6$ nanoparticles per cell). Compared to monocrystalline iron oxide nanoparticle (MION), a prototype clinical preparation, several small-molecule modifications (e.g., iodoacetic anhydride, diaminopropane or diethylenetriamine) showed much lower macrophage uptake, an important consideration for improving *in vivo* targeting. We also noted a considerable difference in cellular uptake between resting and GM-CSF–stimulated human macrophages in the primary screen (**Fig. 2**).

Cell-based phenotyping

To examine whether macrophage activation status could indeed be probed with selected materials, we scaled up the synthesis of two nanoparticle preparations (CLIO-bentri and CLIO-gly) identified through the primary screen. Primary isolated human macrophages were cultured for 7 d and stimulated with GM-CSF (to simulate macrophages in immune disease), oxidized low density lipoprotein (LDL) (to simulate foam cells found in atherosclerosis¹⁷) or lipopolysaccharide (LPS) (to simulate macrophages in infection). CLIO-bentri (compound 261-14-1, Supplementary Table 1) was by far the preferred compound internalized into resting macrophages, with uptake higher than the parent compound CLIO-NH₂, whereas CLIO-gly (compound 261-47-1, Supplementary Table 1) was the preferred compound for activated macrophages (for all three activation methods-GM-CSF, ox-LDL, LPS) as determined by epifluorescence and confocal microscopy (Fig. 3). Interestingly, the starting material CLIO-NH₂ showed no apparent preference among the cell populations. These compounds (Fig. 2) are promising candidates for developing more efficient agents for treating autoimmune diseases¹⁸ or detecting vulnerable atherosclerotic plaques¹⁹.

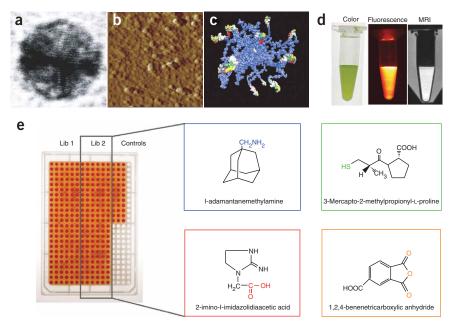


Figure 1 Nanoparticle and derived-nanoparticle library. (**a**,**b**) Laser light scattering (**a**) and atomic force microscopy (**b**) were used to reveal nanoparticles (38 nm mean diameter) comprising a magnetic core and surface-bound, crosslinked dextran. (**c**) Model of the crosslinked dextran coating modified with small molecules. (**d**) The nanoparticles are fully soluble in water, are fluorescent and superparamagnetic, that is, detectable by magnetic resonance imaging (MRI). (**e**) Different classes of small molecules with amino, sulfhydryl, carboxyl or anhydride functionalities were anchored onto the nanoparticles and stored in multiwell plates for testing.

Based on these screening results, we next investigated whether small-molecule modification can impart unique biological functions to nanoparticles. Molecularly targeted nanomaterials show promise for visualizing specific targets in vivo and for delivering therapies. Cell-internalizing affinity ligands are often used to improve targetto-background ratios^{15,20}. We hypothesized that this and other amplification strategies could be further enhanced by decreasing nonspecific macrophage uptake of nanoparticles through modification of their surfaces. After identifying unique nanoparticles with low macrophage uptake (Fig. 2), we asked whether iodoacetate surface modification (shown above to reduce macrophage uptake) could improve target-tomacrophage ratios conferred by a VCAM-1 targeting peptide sequence (VHSPNKK)¹⁵. Figure 4 compares cellular uptake into VCAM-1positive murine heart endothelial cells (MHEC) and nonspecific uptake into VCAM-1-negative murine macrophages. In vivo targeting of activated VCAM-1-expressing endothelial cells present in macrophage-rich diseases such as atherosclerosis requires a reduction in nonspecific nanoparticle phagocytosis into macrophages. As expected, peptide attachment via the C-terminal carboxylate to the unmodified CLIO nanoparticle increased uptake into MHEC, but there was also high uptake into macrophages. Iodoacetic anhydride modification of the peptide-conjugated nanoparticle greatly decreased macrophage uptake. This caused a tenfold improved target-to-background ratio, confirming cellular uptake results. Similar results were also observed for other small molecules that decreased macrophage uptake (Fig. 2).

In vivo imaging

We next determined whether the library could be used to identify compounds that preferentially targeted cancer cells but had concomitantly low uptake in macrophages and endothelial cells. Given the

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 4-amino-1,8-naphthalic anhydride; 13.2 bicycol (2.2) cot-7-ene -2.3,5-6-tetrashdrophthalic anhydride; 166.0 hero-3,6-epoxy-1,2.3,6-tetrashdrophthalic anhydride; 166.0 heromophthalic anhydride; 184.1 Decanoic ahd; 326.5 cis-aconitic anhydride; 181.1 trifluoroacetic anhydride; 181.1 taconic anhydride; 182.1 taconic anhydride; 181.1 taconic anhydride; 182.1 taconic anhydride; 182.2 tachydrawnine; 87.2 tachydrawnine; 183.0 tacing anhydride; 183.1 tacing anhydride; 183.1 tacing anthydrawnine; 184.0 tacing anthydrawnine; 187.0 tacing anthydrawnine; 187.0 taci						3,4,5,6-tetrahydrophthalic anhvdride: 152.2
<pre>1-cyclopentene-1.2-dicarboxy anhydride; 138.1 exo 3.6 epoxy-1.2.3 clearboxy anhydride; 166.0 homophthalic anhydride; 184.1 Decanoic ahd; 326.5 cis-acontic anhydride; 188.1 trifluoroacetic anhydride; 180.1 trifluoroborne-endo-2.3-dicarboxylic anhydride; 164.0 Diglycolic anhydride; 116.1 phthalic anhydride; 116.1 interviewertriaminepentaacetic dianhydride; 357.3 5-chloroisatoic anhydride; 184.1 1.2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 114.1 3-hrito-1.8-naphthalic anhydride; 243.2 1-hexadecylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 70.1 Terr-butylamine; 73.1 4-miro-1.8-naphthalic anhydride; 142.2 Hexylamine; 73.1 4-miro-1.8-naphthalic anhydride; 142.1 3-methylglutaric anhydride; 128.1 isoamylamie; 87.2 2-adiamethylglutaric anhydride; 128.1 isoamylamie; 87.2 2-adiamethylglutaric anhydride; 128.1 isoamylamie; 87.2 2-amethylglutaric anhydride; 128.1 isoamylamie; 87.2 2-amethylglutaric anhydride; 128.1 isoamylamine; 87.2 Hexylamine; 87.0 Hexylamine; 87.2 Hexylamine; 87.0 Hexylamine; 87.2 Hexylamine; 87.0 Hexylamine; 87.2 Hexylamine; 87.2 Hexylamine;</pre>						4-amino-1,8-naphthalic anhydride; 213.2
exo-3.6-epoxy-1.2.3.6-tetratydrophthalic anhydride; 166.0 homophthalic anhydride; 162.1 diflorophthalic anhydride; 158.1 trifluoroactic anhydride; 210.0 Cis-5-norborne-endo-2.3-dicarboxylic anhydride; 164.0 Diglycolic anhydride; 116.1 phthalic anhydride; 117.1 diethylenetriaminepentaacetic dianhydride; 357.3 5-chlorisatio anhydride; 117.1 diethylenetriaminepentaacetic dianhydride; 357.3 5-chlorisatio anhydride; 116.1 1.2-cyclohexanedicarboxylic anhydride; 154.2 glutaric anhydride; 114.1 3-nitro-1.8-napthalic anhydride; 243.2 1-hexadecylamine; 241.3 4-nitro-1.8-napthalic anhydride; 142.2 Hexylamine; 101.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 101.2 Tert-butylamine; 73.1 dimethylgropylamine; 87.2 methysucolic anhydride; 114.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 methysucolic anhydride; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 215.6 L-Giu; 147.0 L-Thr; 119.0 L-Ty; 181.0 L-Vai; 117.0 L-Ty; 181.0 L-Vai; 117.0 L-Ty; 181.0 L-Vai; 117.0 L-Ty; 181.0 L-Vai; 117.0 L-Ty; 181.0 L-Vai; 117.0 L-Ty; 180.0 L-Vai; 117.0 L-Ly; 142.0 L-Kai; 180.0 NiCN CLIO-TAT						bicyclo [2,2,2] oct-7-ene-2,3,5,6-tetracarboxylic anhydride; 248.0
difforophthalic anhydride; 184.1 Decanoic and; 326.5 cis-aconitic anhydride; 116.1 phthalic anhydride; 116.1 phthalic anhydride; 116.1 phthalic anhydride; 117.1 diethylenetriaminepentaacetic dianhydride; 357.3 5-chlorisatic anhydride; 117.6 3-hydroxyphthalic anhydride; 164.1 1.2-cyclohexanedicatroxy anhydride; 164.2 glutaric anhydride; 114.1 3-nitro: 1.8-napthalic anhydride; 243.2 1-hexadecylamine; 241.5 1.4.5,6-naphtaliae anhydride; 142.2 hexanethylolutar anhydride; 142.2 hexanethylolutar anhydride; 142.2 hexanethylolutar anhydride; 142.1 4-nitro: 1.8-napthalic anhydride; 243.2 1-hexadecylamine; 73.1 4-nitro: 1.8-napthalic anhydride; 243.2 1-hexadecylamine; 73.1 4-nitro: 1.8-napthalic anhydride; 142.2 hexanethylolutaric anhydride; 142.1 5-chlorisatic anhydride; 142.1 4-nitro: 1.8-napthalic anhydride; 142.2 hexanethylolutaric anhydride; 142.2 hexanethylolutaric anhydride; 142.2 hexanethylolutaric anhydride; 142.2 hexanethylolutaric anhydride; 142.2 hexanethylolutaric anhydride; 142.1 3-methylopoplamine; 87.2 methysiconic anhydride; 142.1 3-methylopoplamine; 87.2 hexanethylopoplamine;						1-cyclopentene-1,2-dicarboxy annydride; 138.1 exo-3 6-epoxy-1 2 3 6-tetrabydrophthalic anbydride; 166.0
Decanoic ahd; 326.5 cis-aconitic anhydride; 158.1 trifluoroacetic anhydride; 161.0 Cis-5-notorne-endo-2,3-dicarboxylic anhydride; 164.0 Diglycolic anhydride; 116.1 phthalic anhydride; 116.1 laconic anhydride; 116.1 laconic anhydride; 116.1 laconic anhydride; 116.1 l.2-cyclohexanadicarboxy anhydride; 154.2 giutaric anhydride; 114.1 3-hritro-1,8-naphthalic anhydride; 243.2 1-hexadecylamine; 241.5 1,4,5,8-naphtalenettracarboxylic anhydride; 268.2 Amylamine; 70.2 2,2-diamethylgiutaric anhydride; 142.2 Hexylamine; 70.1 4-mitor-1,8-naphthalic anhydride; 243.2 1-ethylgiutaric anhydride; 144.1 3-methylgiutaric anhydride; 142.1 8-methylgiutaric anhydride; 142.2 Hexylamine; 70.2 Tert-butylamine; 73.1 4-mitor-1,8-naphthalic anhydride; 243.2 1-ethylgiutaric anhydride; 144.1 3-methylgiutaric anhydride; 143.1 isoamylamine; 87.2 Hexylamine; 87.2 Hexylamine; 87.2 Hexylamine; 87.2 Hexylamine; 87.2 Hexamethylenediamine; 116.2 2-aminohydride; 115.2 1,4-diaminobutane; 88.2 Ethylenediamine; 10.1 D-Glucoselic anhydride; 144.0 CLO-SUG 4-cholorophenyl alanine; 150.0 L-Yen; 117.0 L-Yen; 117.0						homophthalic anhydride; 162.1
<pre>cis-aconitic anhydride; 158.1 trifluoroacetic anhydride; 210.0 Cis-5-norborne-endo-2,3-dicarboxylic anhydride; 164.0 Diglycolic anhydride; 116.1 phthalic anhydride; 117.1 diethylenetriaminepentaacetic dianhydride; 357.3 5-chloroisatic anhydride; 117.1 1.2-cyclohexanedicarboxylic anhydride; 154.2 glutaric anhydride; 114.1 3-nitro-1,8-napthalic anhydride; 243.2 1-hexadecylamine; 241.5 1.4,5,6-napthalica chrydride; 243.2 1-hexadecylamine; 241.5 1.4,5,6-napthalica chrydride; 243.2 1-hexadecylamine; 87.2 rethylorophamine; 87.2 Hexamethylorophamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 10.0 L-Giu; 147.0 L-Tw; 118.0 L-Vwi; 117.0 L-Tw; 181.0 L-Vwi; 181.0 L-Vwi; 181.0 L-Vwi; 181.0 L-Vwi; 181.0 L-Vwi; 181.0 L-Vwi; 181.0 L-Wwi; 183.0 L-Pm; 185.0 L-Pm; 180.0 L-No; 140.0 L-Ai; 89.0 MiCN CLIO-Tat</pre>						diflorophthalic anhydride; 184.1
 triffuoracetic anhydride; 210.0 Cis-5-notorne-endo-2-3-dicarboxylic anhydride; 164.0 Diglycolic anhydride; 116.1 phthalic anhydride; 118.1 laconic anhydride; 118.1 laterylienetriaminepentaacetic dianhydride; 357.3 5-chloroisatoic anhydride; 118.1 1,2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 114.1 3-nitro-1,8-naphtalic anhydride; 243.2 1-hexadecylamine; 271.5 1,4,5,8-naphtalenetrtracarboxylic anhydride; 268.2 Amydramie; 87.2 2,2-diamethylglutaric anhydride; 142.2 Hexylamine; 73.1 4-nitro-1,8-naphtalic anhydride; 243.2 1-ehxadecylamine; 272.2 2-diamethylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.2 identifylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.1 dimethylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.2 identifylgropylamine; 87.2 Hexamethylglutaric anhydride; 14.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.1 dimethylgropylamine; 87.2 Hexamethylenediamine; 10.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Yr; 119.0 L-Yr; 119.0 L-Yr; 119.0 L-Yr; 119.0 L-Yr; 110.1 L-Ser; 126.0 L-Glucosamine; 137.0 2-adamantanamine; 137.0			-			cis-aconitic anhydride: 158 1
 Diglycolic anhydride; 116.1 phthalic anhydride; 118.1 ltaconic anhydride; 118.1 ltaconic anhydride; 118.1 ltaconic anhydride; 118.1 ltaconic anhydride; 118.1 ltaconic anhydride; 118.1 l.2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 114.1 3-nitro-1.8-naphtalic anhydride; 243.2 1-hexadecylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 87.2 7.2-diamethylglutaric anhydride; 142.2 Hexylamine; 87.2 7.2-diamethylglutaric anhydride; 143.1 3-methylglutaric anhydride; 14.1 3-methylglutaric anhydride; 128.1 isoamylamic; 87.2 isobutylamine; 87.2 Hexamethylenediamine; 116.2 2-aminohytare; 15.2 1.4-diaminobutare; 88.2 Ethylenediamine; 215.6 L-Glucosamine; 150.0 L-Mi; 117.0 L-Mi; 110.0 L-Mi; 117.0 L-Mi; 117.0 L-M						trifluoroacetic anhydride: 210.0
<pre>phthalic anhydride; 148.1 Itaconic anhydride; 112.1 diethylenetriaminepentaacetic dianhydride; 357.3 5-chlorösatoic anhydride; 197.6 3-hydroxyphthalic anhydride; 164.1 1.2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 114.1 3-nitro: 1.8-naphalia enhydride; 243.2 1-hexadecylamine; 241.5 1.4.5,8-naphtaliaentrucarboxylic anhydride; 268.2 Armylamine; 87.2 2.2-diamethylglutaric anhydride; 142.2 therh-duylamine; 73.1 4-nitro: 1.8-naphtaliaentrucarboxylic anhydride; 268.2 Armylamine; 87.2 reth-duylamine; 73.1 4-nitro: 1.8-naphtaliaentrucarboxylic anhydride; 243.2 1-ethylgropylamine; 87.2 methysiconic anhydride; 114.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 Hexamethylglutaric anhydride; 128.1 isoamylamine; 130.0 L-Giu: 140.0 L-As; 130.0 L-He; 130.</pre>						
diethylenetriaminepentaacetic dianhydride; 357.3 5-chlorösatoic anhydride; 197.6 3-hydroxyphthalic anhydride; 164.1 1.2-cyclohexanedicatroxy anhydride; 154.2 glutaric anhydride; 114.1 3-nitro 1.8-napthalica nhydride; 243.2 1-hexadecylamine; 72 2.2-diamethylglutaric anhydride; 142.2 Hexylamine; 70.12 1art-bothylamine; 73.1 dimethylglutaric anhydride; 128.1 isoamylamine; 73.1 dimethylglutaric; 87.2 Hexamethylglutaric; 87.4 Hexamethylglutaric; 87.9 Hexamethylglutaric; 83.9 Hexamethylglutaric; 83.9 Holebyleretiannic; 156.0 Pentaethylglutaric; 83.9 Holebyleretiannic; 156.0 Pentaethyleretiannic; 156.0 Pentaethylglutaric; 83.9 Holebyleretiannic;						Diglycolic anhydride; 116.1 phthalic anhydride: 148.1
 Schloroisatoic anhydride; 197.6 Schydroxypithalic anhydride; 164.1 1,2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 141.1 Schitro.1,8-naphtalic anhydride; 243.2 1-hexadecylamine; 87.2 2,2-diamethylgitutric anhydride; 142.2 Hexylamine; 70.1 Tert-butylamine; 77.1 4-nitro.1,8-naphtalic anhydride; 243.2 1-eixylamine; 77.2 Tert-butylamine; 77.2 Tert-butylamine; 77.2 Hexylamine; 77.2 Hexamethylenediamine; 10.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Tyr; 18.0 L-Tyr; 18.0 L-Glu; 147.0 L-Tyr; 19.0 L-Mexylamine; 150.0 A-droidon antanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 130.0 2-doine; 146.0 L-Mexylamine; 163.1 L-Hix; 155.0 						Itaconic anhydride; 112.1
 3-hydroxyphthalic anhydride; 164.1 1.2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 114.1 3-nitro:1,8-naphtalic anhydride; 243.2 1-hexadecylamine; 241.5 1.4,5,8-naphtalicar tracarboxylic anhydride; 268.2 Amylamine; 73.1 4-nitro:1,8-naphtalic anhydride; 142.2 Hexylamine; 70.1 Tert-butylglutaric anhydride; 142.2 Hexylamine; 73.1 4-nitro:1,8-naphtalic anhydride; 243.2 1-ethylproylamine; 87.2 methylglutaric anhydride; 141.1 3-methylglutaric anhydride; 142.1 Hexylamine; 73.1 4-nitro:1,8-naphtalic anhydride; 243.2 1-ethylpropylamine; 87.2 methylglutaric anhydride; 116.2 2-aminoheptane; 115.2 1.4-diminobutane; 88.2 Ethylenediamine; 73.6 L-Glucsamine; 215.6 L-Glu; 147.0 L-Thr; 19.0 L-H						diethylenetriaminepentaacetic dianhydride; 357.3
 1,2-cyclohexanedicarboxy anhydride; 154.2 glutaric anhydride; 141.1 3-nitro-1,8-naphtalic anhydride; 243.2 1-hexadecylamine; 241.5 1,4,5,8-naphtalenetrtracarboxylic anhydride; 268.2 Amylamine; 87.2 2,2-diamethylgitutaric anhydride; 142.2 Hexylamine; 70.1 4-nitro-1,8-naphtalic anhydride; 243.2 1-ethylpropylamine; 87.2 methysucolic anhydride; 114.1 3-methylgitutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.1 dimethylgitutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.2 Hexylamine; 73.1 dimethylgitutaric anhydride; 128.1 isoamylamic; 87.2 isobutylamine; 87.2 Hexamethylenediamine; 116.2 2-aminohytane; 85.2 Ethylenediamine; 80.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Tyr; 18.0 L-Tyr; 18.0 L-Tyr; 18.0 L-Tyr; 18.0 L-Tyr; 18.0 L-Tyr; 19.0 L-Tyr; 19.0 L-Tyr; 19.0 L-Ser; 105.0 L-Mei; 149.0 L-Phe; 165.0 L-His; 155.0 Dethylenetriamine; 156.0 Pentatehylenetriamine; 156.0 Pentatehylenetriamine; 156.0 Pentatehylenetriamine; 156.0 Pentatehylenetriamine; 156.0 Pentatehylenetriamine; 156.0 Pen						3-hydroxyphthalic anhydride; 197.6
 3-nitro-1,8-naphtalic anhydride; 243.2 1-hexadecylamine; 241.5 1,4,5,8-naphtalenettracarboxylic anhydride; 268.2 Amylamine; 87.2 2,2-diamethylgittaric anhydride; 142.2 Hexylamine; 70.1 4-nitro-1,8-naphtalic anhydride; 243.2 1-ethylpropylamine; 87.2 Inethylgittaric anhydride; 141.1 3-methylgittaric anhydride; 128.1 isoamylamine; 87.2 Isobutylamine; 73.1 dimethylgittaric anhydride; 128.1 isoamylamine; 87.2 Isobutylamine; 73.2 Hexylamine; 73.1 dimethylgittaric anhydride; 128.1 isoamylamine; 87.2 Isobutylamine; 73.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 215.6 L-Giu; 147.0 L-Trr; 181.0 L-Vai; 117.0 L-Tri; 181.0 L-Vai; 117.0 L-Tri; 181.0 L-Vai; 117.0 L-Tri; 181.0 L-Vai; 117.0 L-Tri; 181.0 L-Asi; 128.0 L-Ser; 105.0 L-Mei; 149.0 L-Phe; 165.0 L-Phe; 165.0 L-Phe; 165.1 L-Phe; 163.1 pentadecylamine; 129.0 L-Phe; 149.0 L-Phe; 163.1 pentadecylamine; 156.0 Pentaterylamine; 156.0 Pentaterylenetria; 215.0 L-Phe; 163.1 Diethylenetria; 156.0 L-Phe; 163.1 Diethylenetria; 156.0 Pentaterylenetria; 156.0 Pentaterylenetria						1,2-cyclohexanedicarboxy anhydride; 154.2
1,4,5,4-naphtalenetrtracarboxylic anhydride; 268.2 Amylamine; 87.2 2,2-diamethylglutaric anhydride; 142.2 Hexylamine; 70.1 4-nitro:1,8-naphtalic anhydride; 243.2 1-ethylpropylamine; 87.2 methysuccinic anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylgropylamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosarnine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Thr; 129.0 L-Thr; 129.0 L-Thr; 129.0 L-Phe; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 120.0 L-Ma; 129.0 MICN CLIO-TAT						glutaric anhydride; 114.1
1,4,5,4-naphtalenetrtracarboxylic anhydride; 268.2 Amylamine; 87.2 2,2-diamethylglutaric anhydride; 142.2 Hexylamine; 70.1 4-nitro:1,8-naphtalic anhydride; 243.2 1-ethylpropylamine; 87.2 methysuccinic anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylglutaric anhydride; 114.1 3-methylgropylamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosarnine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Thr; 129.0 L-Thr; 129.0 L-Thr; 129.0 L-Phe; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 129.0 L-Dhr; 120.0 L-Ma; 129.0 MICN CLIO-TAT						1-hexadecvlamine: 241.5
2,2-diamethylglutaric anhydride; 142.2 Hexylamine; 101.2 Tert-butylamine; 73.1 4-nitro: 1.8-naphthalic anhydride; 243.2 1-ethylpropylamine; 87.2 methysuccinic anhydride; 114.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 87.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Thr; 129.0 L-Thr; 129.0 L-Thr; 110.0 L-Thr; 20.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-TAR; 140						1,4,5,8-naphtalenetrtracarboxylic anhydride; 268.2
 Inter-Dutylamine; 73.1 A-nitor-1, 8-naphthalic anhydride; 243.2 I-ethylpropylamine; 87.2 Inethylpropylamine; 87.2 Isoamylamine; 87.2 Isobutylamine; 73.1 dimethylpropylamine; 87.2 Isobutylamine; 73.1 dimethylpropylamine; 87.2 Hexamethylenetiamine; 116.2 2-aminoheptane; 115.2 I-diaminobutane; 86.2 Ethylenetiamine; 215.6 L-Giu; 147.0 L-Tyr; 187.0 L-Giu; 146.0 L-Giu; 146.0 L-Giu; 146.0 L-Denylaanine; 150.0 L-Ag; 174.2 L-Ag; 174.2 L-Ag; 174.5 L-His; 155.0 L-Pherphylanine; 215.0 L-Pherphylanine;						Amylamine; 87.2 2.2-diamethylalutaric anhydride: 142.2
 Inter-Dutylamine; 73.1 A-nitor-1, 8-naphthalic anhydride; 243.2 I-ethylpropylamine; 87.2 Inethylpropylamine; 87.2 Isoamylamine; 87.2 Isobutylamine; 73.1 dimethylpropylamine; 87.2 Isobutylamine; 73.1 dimethylpropylamine; 87.2 Hexamethylenetiamine; 116.2 2-aminoheptane; 115.2 I-diaminobutane; 86.2 Ethylenetiamine; 215.6 L-Giu; 147.0 L-Tyr; 187.0 L-Giu; 146.0 L-Giu; 146.0 L-Giu; 146.0 L-Denylaanine; 150.0 L-Ag; 174.2 L-Ag; 174.2 L-Ag; 174.5 L-His; 155.0 L-Pienylaanine Methyl Ester; 179.0 L-Pienylaanine; 213.4 Lodoacetic anhydride; 253.9 L-Seri, 165.0 Pietdeevylamine; 156.0 Pentaethylenethaxamine; 156.0 Pent						Hexylamine; 101.2
 1-ethylpropylamine; 87.2 methysucchic anhydride; 114.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.1 dimethylpropylamine; 87.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Wai; 117.0 L-Tw; 118.10 L-Wai; 117.0 L-Tw; 118.0 L-Asp; 133.0 L-Glu; 146.0 CLIO-SUC 3-Noradamantanamine; 130.0 2-adramatianamine; 150.0 L-Asp; 136.0 L-Glu; 146.0 CLIO-SUC G. L-Bitti 155.0 L-His; 165.0 L-His						Tert-butylamine; 73.1
methysuccinic anhydride; 114.1 3-methylglutaric anhydride; 128.1 isoamylamine; 87.2 isobutylamine; 73.1 dimethylpropylamine; 87.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Thr; 19.0 L-Thr; 19.0 L-Thr; 19.0 L-Thr; 19.0 L-Vai; 17.0 Padamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-damantanamine; 220.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Ma; 180.0 MION CLIO-TAT						
 isobutylamine; 73.2 isobutylamine; 73.2 isobutylamine; 73.2 isobutylamine; 73.2 Hexamethylenediamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Giucosamine; 215.6 L-Giu; 147.0 L-Thr; 119.0 L-Thr; 119.0 L-Vi; 117.0 I-Adamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-damantanamine; 138.0 1.3-diaminopropane; 74.1 Diethylenetriamine; 156.0 2-daminopropane; 74.1 Diethylenetriamine; 156.0 2-daminopropane; 74.1 Diethylenetriamine; 156.0 2-daminopropane; 74.1 Diethylenetriamine; 156.0 2-damantanamine; 232.0 3-hydroxytyramine; 156.0 3-hydroxytyramine; 156.0<						methysuccinic anhydride; 114.1
dimethylproplamine; 87.2 Hexamethylenetiamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenetiamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Thr; 119.0 L-Thr; 119.0 L-Vai; 117.0 Hadamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 220.0 2-adamantanamine; 232.0 2-adamantanamine; 232						3-methylglutaric anhydride; 128.1
dimethylproplamine; 87.2 Hexamethylenetiamine; 116.2 2-aminoheptane; 115.2 1.4-diaminobutane; 88.2 Ethylenetiamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Thr; 119.0 L-Thr; 119.0 L-Thr; 119.0 L-Vai; 117.0 Hadamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 220.0 2-adamantanamine; 232.0 2-adamantanamine; 232						isobutylamine; 73.1
2-aminoheiptane; 115.2 1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 0-Glucosamine; 215.6 1-Glu; 147.0 1-Thr; 119.0 1-Thr; 119.0 1-Vai; 117.0 1-dadamantanemitylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-damantanamine; 137.0 1-Arg; 174.2 1-Asg; 133.0 1-Gin; 146.0 1-Arg; 174.2 1-Asg; 130.0 1-Gin; 146.0 1-Ser; 105.0 1-Fhe; 155.0 1-Fhe; 155.0 1-Pheretriamine; 213.4 1-doacetic anhydride; 353.9 1.3-diaminopropane; 74.1 Diethylenetriamine; 156.0 3-Hydroxytyramine; 156.0 3-Hydroxytyramine; 156.0 2-Bermine; 202.0 N, N'-Bis (2-aminoetryl)-1.3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 1-Ais; 89.0 MION CLIO-TAT						dimethylpropylamine; 87.2
1.4-diaminobutane; 88.2 Ethylenediamine; 60.1 D-Glucosamine; 215.6 L-Glu; 147.0 L-Tyr; 19.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 181.0 L-Vi; 174.2 L-Asp; 133.0 L-Gli; 146.0 L-Gli, 140.0 L-Gli, 140.						
Ethylenediamine; 60.1 D-Giucosamine; 215.6 L-Giu; 147.0 L-Thr; 119.0 L-Thr; 181.0 L-Vai; 117.0 Hadamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 137.0 2-benytalanine Methyl Ester; 179.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 129.0 1-aterzdecylamine; 220.0 1-aterzdecylamine; 250.0 2-benytynamine; 156.0 2-benytynamine; 156.0 2-benytynamine; 156.0 2-benytynamine; 250.0 N. N'-Bis (2-aminoetryl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Aia; 39.0 MICN CLIO-TAT						1.4-diaminobutane: 88.2
L-Glu; 14.7.0 L-Thr; 19.0 L-Thr; 19.0 L-Thr; 181.0 L-Va; 117.0 L-Va; 117.0 L-Va; 117.0 L-Va; 117.0 L-Va; 174.2 L-Asp; 133.0 L-Gin; 146.0 L-Asp; 133.0 L-Gin; 146.0 L-Ser; 105.0 L-Phe; 165.0 L-Phe; 165.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 129.0 L-Phe; 129.0 L-Phe; 129.0 L-Phe; 129.0 L-Phe; 129.0 L-Phe; 129.0 L-Latradecylamine; 213.4 Lodaacetic anhydride; 353.9 1,3-diaminopropane; 74.1 DietHylenetriamine; 110.0 L-U; Sy; 146.0 J-DietHylenetriamine; 156.0 Pentaethylenehexamine; 232.0 Spermine; 202.0 N. N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly: 75.0 Tyramine; 140.0 L-Aa; 39.0 MION CLIO-TAT						Ethylenediamine; 60.1
L-Thr; 119.0 L-Ty; 181.0 L-Val; 117.0 Padamantanemethylamine; 164.0 CLIO-SUC 2-adornantanemethylamine; 161.0 L-Ag; 174.2 L-Asp; 174.2 L-Asp; 174.2 L-Asp; 133.0 L-Gin; 146.0 4-cholorophenyi alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 105.0 L-L-Phe; 105.0 L-Phe; 105.0 L-Phe; 105.0 L-Dependence: 222.0 Spermine; 202.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Pha; 140.0 L-						D-Glucosamine; 215.6
L-1yr; 181.0 L-Va; 117.0 Hadamantanemethylamine; 164.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 137.0 2-adamantanamine; 130.0 L-Asp; 133.0 L-Gin; 146.0 4-cholorophenyl alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 165.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 165.0 L-Phe; 165.0 L-Phe						L-Thr: 119.0
I-adamantanemethylamine; 184.0 CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 130.0 L-Asp; 133.0 L-Gin; 146.0 L-Ser; 105.0 L-Phe; 155.0 L-His; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 155.0 L-Phe; 129.0 L-Pherytalanine Methyl Ester; 179.0 L-Pherytalanine; 220.0 L-Pherytalanine; 213.4 Lodacetic anhydride; 353.9 1,3-diaminopropane; 74.1 Diettylenetriamine; 156.0 Pentaethylenetriamine; 156.0 Pentaethylenetriamine; 156.0 Pentaethylenetriamine; 156.0 Pentaethylenetriamine; 156.0 Pentaethylenetriamine; 160.0 Giy, 75.0 Tyramine; 140.0 L-Ais; 89.0 MION						L-Tyr; 181.0
CLIO-SUC 3-Noradamantanamine; 137.0 2-adamantanamine; 150.0 L-Arg; 174.2 L-Asp; 133.0 L-Gin; 146.0 4-cholorophenyl alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 155.0 L-His; 155.0 L-His; 157.0 L-His; 157.0 L-Lis; 167.0 L-Lis; 16						I-adamantanemethylamine: 164.0
 3-Noradamantanamine; 137.0 2-adamantanamine; 157.0 2-adamantanamine; 150.0 L-Ag; 174.2 L-Ag; 133.0 L-Gin; 146.0 4-cholorophenyl alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-His; 155.0 L-His; 155.0 L-Met; 149.0 L-Pherylalanine Methyl Ester; 179.0 L-Pherylalanine Methyl Ester; 179.0 L-Pherylalanine Methyl Ester; 129.0 L-Etradecylamine; 213.4 Iodacecylamine; 213.4 Iodacecylamine; 156.0 Phenylayvtramine; 156.0 Pentaethylenetriamine; 156.0 Pentaethylenetriamine; 156.0 Spermine; 202.0 N. N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy, 75.0 Tyramine; 140.0 L-Ais; 38.0 MION CLIO-TAT 						CLIO-SUC
L-Arg; 174.2 L-Ag; 174.2 L-Ag; 133.0 L-Gin; 146.0 4-cholorophenyi alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 165.0 L-His; 155.0 L-Met; 149.0 L-Pheryilalanine Methyl Ester; 179.0 L-Pheryilalanine (23.1 D-L-Pheryilalanine; 213.4 Lodaacetic anhydride; 353.9 1,3-diaminopropane; 74.1 Diethylenetriamine; 156.0 Pentaethylenehexamine; 232.0 Spermine; 202.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Aia; 89.0 MION CLIO-TAT						3-Noradamantanamine; 137.0
L-Asp; 133.0 L-Gir; 146.0 4-cholorophenyi alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 165.0 L-He; 165.0 L-He; 155.0 L-He; 149.0 L-Phenyialanine Methyl Ester; 179.0 L-Trp; 204.0 isatoic anhydride; 163.1 pentadecylamine; 213.4 lodoacetic anhydride; 353.9 1.4-diaminopropane; 74.1 Diethylenetriamine; 111.0 L-Lys; 146.0 3-shydroxytyramine; 202.0 Spermine; 202.0 N, N-2Bi (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Aia; 98.0 M(ON CLIO-TAT						L-Arg: 174.2
4-cholorophenyl alanine; 200.0 CLIO-SIA L-Ser; 105.0 L-Phe; 165.0 L-His; 155.0 L-His; 157.0 L-Phenylalanine Methyl Ester; 179.0 L-Th; 204.0 isatoic anhydride; 163.1 pentadecylamine; 227.4 2-Ethylhexylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 219.0 1-tertadecylamine; 220.0 Spermine; 202.0 N, N'-Bis (2-aminochyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Ala; 89.0 M(N) CLIO-TAT						L-Asp; 133.0
CLIO-SIA L-Ser; 105.0 L-Phe; 105.0 L-Phe; 155.0 L-Met; 149.0 L-Pherytalanine Methyl Ester; 179.0 L-Pherytalanine Methyl Ester; 179.0 L-Pherytalanine Methyl Ester; 179.0 L-Pherytalanine; 213.4 Josephilie State Stat						L-Gin; 146.0
L-Ser; 105.0 L-Phe; 155.0 L-His; 155.0 L-His; 155.0 L-His; 157.0 L-Phenylalanine Methyl Ester; 179.0 L-Trp; 204.0 isatoic anhydride; 163.1 pentadecylamine; 227.4 2-Ethylhexylamine; 129.0 1-tetradecylamine; 121.4 lodoacetic anhydride; 353.9 1.3-diaminopropane; 74.1 Diethylenetriamine; 111.0 L-Lys; 146.0 3-hydroxytyramine; 156.0 9-Pintaethylenehoxamine; 232.0 Spermine; 202.0 N, N-EBi (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Ala; 89.0 M(N) CLIO-TAT					-	
L-His; 155.0 L-Met; 149.0 L-Phenylalanine Methyl Ester; 179.0 L-Trp; 204.0 isatoic anhydride; 163.1 pentadecylamine; 129.0 1-tetradecylamine; 129.0 1-tetradecylamine; 121.4 lodoacetic anhydride; 353.9 1.3-diaminopropar; 74.1 Diethylenetriamine; 111.0 L-Lys; 146.0 Phytoxylyamine; 156.0 Phytoxylyamine; 156.0 Phytoxylyamine; 156.0 Phytoxylyamine; 156.0 Phytoxylyamine; 156.0 Phytoxylyamine; 122.0 Semine; 202.0 N, W-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Ala; 89.0 M(NN CLIO-TAT						L-Ser; 105.0
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L-Phenylalanine Methyl Ester; 179.0 L-Trp; 204.0 isatoic anhydride; 163.1 pentadecylamine; 129.0 1-tetradecylamine; 129.0 1-tetradecylamine; 121.4 lodoacetic anhydride; 353.9 1,3-diaminopropar; 74.1 Diethylenetiramine; 111.0 L-Lys; 146.0 3-hydroxyhyramine; 156.0 Pentaattylenetixamine; 232.0 Spermine; 202.0 N, W-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-JA; 89.0 MICN CLIO-TAT						L-Met; 149.0
isatoic anhydride; 163.1 pentadecylamine; 227.4 2-Ethylhexylamine; 129.0 1-tetradecylamine; 129.0 1-tetradecylamine; 121.4 lodoacetic anhydride; 353.9 1.3-dieminopropare; 74.1 Diethylenetriamine; 111.0 2-Lys; 146.0 3-thydroxytyrachine; 156.0 9-chiaethylenetriamine; 232.0 Sper E; 22.0 N, N = Big (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 7 Jyramine; 140.0 L-Ala; 89.0 M(ION CLIO-TAT						L-Phenylalanine Methyl Ester; 179.0
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1-tetradecylamine; 213.4 lodoacetic anhydride; 353.9 1,3-diaminopropane; 74.1 Diethylenetriamine; 111.0 L-Lys; 146.0 3-hydroxytyramine; 156.0 Pentaethylenehexamine; 232.0 Spermine; 202.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Aia; 89.0 MiON CLIO-TAT						pentadecylamine; 227.4
lodoacetic anhydride; 353.9 1.3-diaminopropane; 74.1 Diethylenetriamine; 111.0 L-Lys; 146.0 3-hydroxytyramine; 156.0 Pentaettylenehexamine; 232.0 Spermine; 202.0 N, V-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Giy; 75.0 Tyramine; 140.0 L-Ala; 89.0 MICN CLIO-TAT						2-Ethylhexylamine; 129.0
Diethylenetriamine; 111.0 L-Lys; 146.0 3-hydroxytyramine; 156.0 Peritaethylenehexamine; 232.0 Spermine; 202.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-Ala; 89.0 MICN CLIO-TAT						Iodoacetic anhvdride: 353.9
Diethylenetriamine; 111.0 L-Lys; 146.0 3-hydroxytyramine; 156.0 Peritaethylenehexamine; 232.0 Spermine; 202.0 N, N'-Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-Ala; 89.0 MICN CLIO-TAT						1,3-diaminopropane; 74.1
N, N -Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-Ala; 83.0 MION CLIO-TAT						Diethylenetriamine: 111.0
N, N -Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-Ala; 83.0 MION CLIO-TAT						3-hydroxytyramine; 156.0
N, N -Bis (2-aminoethyl)-1,3-propanediamine; 160.0 Gly; 75.0 Tyramine; 140.0 L-Ala; 83.0 MION CLIO-TAT						Pentaethylenehexamine; 232.0
Giy; 75.0 Tyramine; 140.0 L-Ala; 89.0 MION CLIO-TAT						N. N' -Bis (2-aminoethyl)-1.3-propanediamine: 160.0
L-Ala; 89.0 MION CLIO-TAT						Gly; 75.0
MION CLIO-TAT						ryramine; 140.0 L-Ala: 89.0
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Figure 2 Heat map representing cellular uptake of different nanoparticle preparations. Columns from right to left: 1, pancreatic cancer cells (PaCa-2); 2, macrophage cell line (U937); 3, resting primary human macrophages; 4, activated primary human macrophages; 5, human umbilical vein endothelial cells (HUVEC). Each column represents mean values from six different experiments. Red refers to the lowest accumulation and green refers to the highest accumulation.

lack of efficient molecules for early detection of pancreatic cancer, we selected PaCa-2 cells from this tumor-type as a model system (**Fig. 2**). Fourteen compounds showed significant uptake into these cancer cells (up to 160×10^6 nanoparticles per cell for the most efficient compounds).

Of these compounds, two (isatoic anhydride, 261-15-28 and 5-chloro-isatoic anhydride, 261-14-17) exhibited high cancer cell uptake and low macrophage/endothelial cell uptake. These compounds were scaled up for in vivo use and shown to facilitate pancreatic cancer detection in a mouse model (Fig. 5). Simultaneous injection of CLIO-NH2-Cy3.5 and CLIO-isatoic-Cy5.5 into tumor-bearing mice significantly increased fluorescence of CLIO-isatoic-Cy5.5 versus CLIO-NH₂-Cy3.5 (TBR 1.63 vs. 0.16, P < 0.0001) as determined by fluorescence imaging. These findings were corroborated by fluorescence microscopy showing widespread accumulation of targeted nanoparticles within tumor cells, indicating access to the tumor interstitium through capillaries. Additional in vivo experiments demonstrated that the signal in the Cy5.5 channel arose primarily from targeting and not from enhanced photon propagation at different wavelengths. Figure 6 shows in vivo targeting in different channels depending on different fluorochromes covalently attached to CLIO-isatoic.

To verify the fluorescent screening data, we also conducted quantitative biodistribution experiments with ¹¹¹In-labeled nanoparticles, bearing in mind that further diethylene triamine pentaacetic acid modification might mitigate isatoic-mediated targeting effects (Fig. 5). A higher uptake of the targeted nanoparticle preparation was seen in pancreatic cancer cells $(3.7 \pm 0.14 \text{ injected dose (ID)/g for})$ CLIO-isatoic versus 2.2 \pm 0.39 ID/g for CLIO-NH₂, P < 0.0001; Fig. 5), whereas uptake in liver (40.4 \pm 5.3 ID/g for CLIO-isatoic versus 40.2 \pm 3.6 ID/g for CLIO-NH₂), lung, muscle and other organs was similar for both preparations (Fig. 5). Enhanced uptake in liver is commonly observed with carbohydrate-modified nanoparticle preparations because of the efficient uptake mechanism into cells of the reticuloendothelial system and the large blood volume and high perfusion of the liver. Although this may pose limitations to in vivo imaging (particularly with isotope- and fluorochrome-labeled nanoparticles), it may be less of a problem with spatially resolved techniques such as magnetic resonance imaging. It is conceivable that library approaches similar to the one described here may be used to discover compounds with lower liver accumulation. Overall, these results indicate that small-molecule modification can indeed impart unique functions to nanoparticles and facilitate in vivo targeting.

DISCUSSION

We have applied a magnetofluorescent nanoparticle library decorated with small molecules to address four critical questions in biomedical nanomaterial research: (i) Is it possible to modulate the relative affinity of nanoparticles for different cell types through small-molecule surface modification? (ii) Can small-molecule surface modification be used to discriminate between closely related functional states of cells? (iii) Can affinity tag-conjugated nanomaterials be made more efficient by reducing macrophage uptake? and (iv) Can disease-specific nanoparticles be developed without a priori knowledge of the target? We show several proof-of-principle examples attesting to the ability of small-molecule modifications to address these questions. Screening of this library identified a variety of nanomaterials that discriminate among different cell types as well as among different physiologic states of the same cell type. We envision that similar high-throughput screens could facilitate other tasks such as identifying the effects of nanomaterials on cell differentiation²¹, toxicity²² and pharmacokinetics. Furthermore, attachment of small molecules to magnetofluorescent

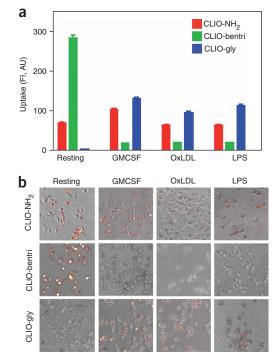


Figure 3 Nanoparticle 'hits' identified from the large screen (Fig. 2) were probed against resting and activating macrophages. (a) Quantitative fluorescence-activated cell sorting (FACS) analysis from three separate experiments. (b) Epifluorescence microscopy with 680-nm channel (CLIO-Cy5.5) merged onto phase contrast images. Note the preference of CLIObentri for resting macrophages and CLIO-gly for activated macrophages. Notably, the starting material CLIO-NH2 shows no apparent preference among the cell populations (mean \pm s.d.). GM-CSF, granulocyte macrophage colony; stimulating factor; Ox-LDL, oxidized low density lipoprotein; LPS, lipopolysaccharide. Scale bar, 10 μ m.

nanoparticles allows efficient identification of binders that would otherwise be difficult to recover using small-molecule screens.

The small molecules used here were chosen to enable rapid synthesis and surface modifications. We believe that the efficacy of the described materials may be attributed to the multivalent nature of the surface molecules (60 ligands per nanoparticle). Multivalent interactions occur throughout biology and represent an evolutionary trend that exploits an existing pool of interactions rather than developing new ones. Multivalent drug design has yielded anti-inflammatory and antiviral agents several orders of magnitude more potent than monovalent agents²³. Our results are in line with these observations and show that nanoparticle uptake can be increased by several orders of magnitude.

A logical extension of this work would be the attachment of novel complex small molecules with defined biological properties and specific protein binding²⁴. Recent advances in diversity-oriented synthesis have allowed the creation of libraries of molecules that resemble natural products in their complexity and possess significant skeletal and stereochemical diversity²⁴. Attachment of such libraries to nanoparticles in array could further extend the diversity of nanomaterials. Finally, the approach could be used not only to screen for novel binders but also to optimize linkers, spacers and conjugation chemistry, and to modulate pharmacokinetics and recognition by the reticuloendothelial system (e.g., for uptake in liver and spleen). Although this study used only one type of biocompatible nanoparticle

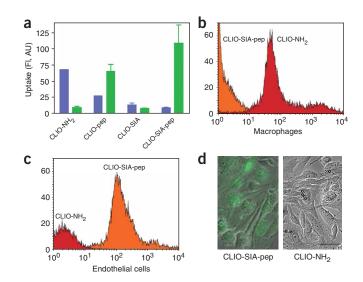


Figure 4 Targeting experiments. (a) Cellular uptake of different nanoparticle preparations into macrophages lacking VCAM-1 (blue) or murine heart endothelial cells (MHEC) expressing VCAM-1 (green). The starting material CLIO-NH₂ shows a preferential affinity for macrophages, whereas SIA modification decreases macrophage affinity. VHS peptide conjugation to CLIO-NH₂ increases MHEC uptake whereas combined peptide attachment and SIA modification results in the highest levels for VCAM-1 specific targeting. (b) FACS analysis of the starting material CLIO-NH₂ and the combined effects of peptide attachment and SIA modification of CLIO-NH₂ in macrophages. (c) FACS analysis of the starting material CLIO-NH₂ and the combined effects of peptide attachment and SIA modification of CLIO-NH₂ in endothelial cells. (d) Epifluorescence microscopy of VCAM-1–positive murine heart endothelial cells with fluorescence channel merged onto phase contrast images (mean \pm s.d.). Scale bar, 10 μ m.

preparation (dextran-coated nanoparticles), it is likely that the approach would work with other types of nanoparticle preparations as long as biocompatibility, appropriate pharmacokinetics and a capacity for multivalency are ensured. A number of synthetic biocompatible nanoparticles that could serve as base materials for new libraries have recently been described²¹. At least 5 (and preferably 20–100) such functionalities should be incorporated into the design to harness the multivalency of small ligands²³.

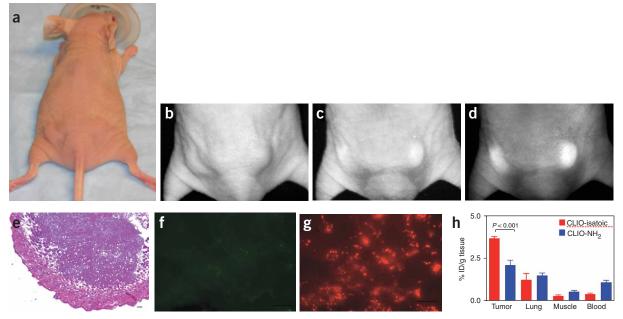
The application of such small-molecule approaches to generating nanomaterials offers the promise of generalized, higherthroughput methods to make novel nanomaterials useful in the diagnosis and treatment of disease. We expect that this union between nanotechnology and small-molecule chemistry will lead to the development of a wide range of novel nanomaterials for biomedical applications.

METHODS

Chemicals. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), sulfo-NHS (sulfosuccinimidyl ester), SPDP (N-succinimidyl 3-(2pyridyldithio) propionate) and SIA (succinimidyl iodo acetate) were purchased from Pierce. Cy5.5 NHS ester was obtained from Amersham. All other chemicals were purchased from Sigma Aldrich and used as received.

Nanoparticle synthesis. The nanoparticle used in this study was a monocrystalline magnetic nanoparticle²⁵, with a 3-nm core of $(Fe_2O_3)_n(Fe_3O_4)_m$ covered with a layer of 10 kDa dextran, that was cross-linked with epichlorohydrin and aminated by reaction with ammonia to provide primary amine groups (CLIO-NH₂)²⁶. The nanoparticle had an overall size (volume weighted)

ARTICLES

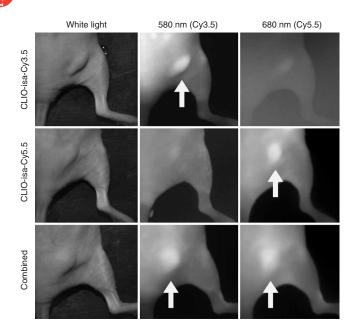


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Figure 5 *In vivo* targeting experiments. (**a**–**h**) PaCa-2 tumors were implanted bilaterally into the hind flanks of nude mice. Mice were injected intravenously with CLIO-Cy3.5 and CLIO-isoatoic-Cy5.5 (1 mg/kg). (**b**–**d**) White light excitation (**b**), Cy3.5 fluorescence channel (**c**) (recording on CLIO-Cy3.5) and Cy5.5 fluorescence channel (**d**) (recording on CLIO-isoatoic-Cy5.5) generated raw black and white images. Note the prominent accumulation of CLIO-isoatoic-Cy5.5 in the bilateral pancreatic tumors indicating tumoral targeting. Hemotoxylin eosin–stained sections of the tumor (**e**). Cryotome sections observed using the Cy3.5 fluorescence channel indicate near-baseline levels of CLIO-Cy3.5 (**f**). Cryotome sections observed using the Cy5.5 fluorescence channel indicate marked fluorescence of CLIO-isatoic within tumor cells (**g**). Biodistribution study with ¹¹¹In-labeled nanoparticles confirms tumoral targeting of CLIO-isatoic (**h**). Scale bar, 10 μm.

in aqueous solution of 38 nm, an R1 of 21 mMsec⁻¹, an R2 of 62 mMsec⁻¹ (37 °C, 0.5 T) and had an average of 62 primary amines available for conjugation. Fluorescein isothiocyanate (FITC) or Cy5.5 NHS ester was dissolved in DMSO and reacted with CLIO-NH₂ to yield an average of two fluorochromes per nanoparticle. The final reaction product was purified on Sephadex G-25 columns and used for small-molecule conjugation.

Conjugation of small molecules. To conjugate anhydrides to $CLIO-NH_2$, 100 µl (50 mM) of the anhydride DMSO solution was added to 2.0 mg CLIO-NH₂ (200 µl of 5.0 mg Fe/ml) in citrate solution. This was followed by



addition of 10 µl 1-M NaOH solution. To conjugate carboxyls, the CLIO-NH2 solution was first exchanged with morpholinoethanesulfonic acid buffer, pH 6.0. The solution was then concentrated to 5.0 mg/ml. We added 100 µl (50 mM) carboxylic acid compound in DMSO to 200 µl of a CLIO- NH_2 MES solution. This was followed by the addition of 5 µmol EDC and 5 µmol sulfo-NHS in MES solution. To conjugate thiols, CLIO-NH2 was first reacted with SPDP. We mixed 1.0 mg SPDP-derivatized CLIO-FITC in 200 µl PBS buffer, pH 7.4 with 100 µl thiol compound in DMSO (50 mM). To conjugate amines (including amino acids), CLIO-NH2 was first reacted with succinic anhydride and purified by Sephadex G-25. We then prepared 200 µl aliquots containing 1.0 mg Fe in MES buffer, pH 6.0 and 10 mg EDC and 10 mg sulfo-NHS was added to this solution. All of the above reactions were allowed to proceed for 2-4 h 21 °C to maximize conversion of all amines. Unreacted small molecules were removed using Sephadex G-25 columns eluted with PBS buffer, pH 7.4. All materials were characterized by size measurements, relaxometry, amine content and mass spectrometry. For biodistribution studies, CLIO-NH2 was modified with three to five DTPA groups per nanoparticle and subsequently modified with small molecules. The compounds were labeled with ¹¹¹In and purified to >99%.

Cells. U937 and PaCa-2 cells were obtained from the American Type Tissue Culture Collection (ATCC) and maintained according to ATCC protocols. For differentiation into macrophages, the nonadherent monocyte-like undifferentiated U937 cells were induced to differentiate by a 48-h exposure to 40 nM

Figure 6 *In vivo* targeting experiments similar to those described in **Figure 5** but with different fluorochrome-labeled nanoparticles (same acquisition parameters in each column). Note the accumulation of CLIO-isoatoic in the tumors irrespective of the fluorochrome used (arrows). Top row: CLIO-isatoic-Cy3.5, middle row: CLIO-isatoic-Cy5.5, bottom row: combined injection of CLIO-isatoic-Cy3.5 and CLIOisatoic-Cy5.5.

phorbol-12-myristate-13-acetate (Sigma). After addition of PMA, cells were plated onto gelatin-coated 96-well tissue culture plates. Differentiated cells were maintained by replacement of PMA-containing media every 2–3 d.

Primary human macrophages were obtained from buffy coats. Briefly, mononuclear cells were isolated by density centrifugation using lymphocyte separation media (LSM; ICN Biomedicals) and the MACS human monocyte isolation kit (Miltenyi Biotec). Monocytes were plated onto cell culture-treated dishes and cultured in primary human monocyte medium containing RPMI 1640 1× with 10% fetal bovine serum, 10 ml/L 200 mM L-glutamine and 10 ml/L 10,000 IU/ml penicillin-streptomycin. Freshly isolated monocytes were treated to produce resting macrophages, GM-CSF-activated macrophages, foam cells and lipopolysaccharide S (LPS)-activated macrophages. To produce resting macrophages, cells were fed every 2-3 d while in culture. After 7 d, adherent cells were considered resting macrophages. GM-CSF-activated macrophages were prepared as follows: GM-CSF (stock solution 1 µg/ml) (BD Biosciences) was added to primary human monocyte medium at a dilution of 12 µl of GM-CSF solution per 1.0 ml of medium. The cells were maintained at this concentration of GM-CSF for 7 d before use in experiments. Foam cells were generated by treating primary human monocyte-derived macrophages with 10 µl of a 2 mg/ml stock solution of oxidized low-density lipoprotein (oxLDL, Biomedical Technologies) for 7 d before experiments. In addition, macrophages were treated with LPS for 24 h.

Screening. All cells were plated in 96-well 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% CO₂. After incubation, wells were washed $3\times$ with PBS/0.1% BSA/0.05% Tween-20 wash buffer and then analyzed using fluorescence microscopy, flow cytometry or an immunoassay to quantify FITC concentration²⁷. Microscopy was done using a Biorad 2000 confocal microscope or a Nikon 80i Eclipse microscope equipped with a 512 Photometrics Cascade CCD. Flow cytometry was performed using a Becton Dickinson FACSCalibur. All experiments were performed at least six times.

For each screening experiment, the uptake of FITC (pM) was log_{10} transformed, and the mean determined for each cell line. The resulting data were centered on the median and normalized by the standard deviation. The compounds were clustered with Cluster 3.0 using average linkage, and Euclidean distance as the similarity metric²⁸. The heat map and the dendrogram of the clustered data were visualized using Java TreeView²⁹. The color scale ranged from green (highest uptake) to black to red (lowest uptake).

In vivo experiments. Selected fluorescence-labeled compounds were tested in a pancreatic adenocarcinoma xenograft mouse model after intravenous administration of different nanoparticle preparations (1 mg Fe/kg nanoparticle; n = 16 mice). Cohorts of mice received either (i) CLIO-isatoic-Cy3.5, (ii) CLIO-isatoic-Cy5.5 or (iii) mixtures of CLIO-isatoic-Cy3.5 and CLIO-isatoic-Cy5.5. Twenty-four hours later, animals were imaged using a fluorescence-imaging system (BonSai, Siemens) at different wavelengths, and values were expressed as ratios between the fluorochromes¹⁰. Tumoral fluorescence was calculated as tumor – background/background and values among different groups were compared using Student's *t*-test. Biodistribution experiments of DTPA-CLIO-NH₂-Cy3.5 and DTPA-CLIO-isatoic-Cy5.5 were performed in PaCa2-bearing mice (n = 10) following intravenous administration of ¹¹¹In-labeled compounds (100 µl, 100 µCi/mouse). Organ distribution was expressed as % injected dose/g tissue.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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