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Application Note 5.

Title. Receptor mediated endocytosis: Conjugation of spermidine to **CL-30Q02-6C**, a carboxyl containing USPIO. JRH/EVG

Keywords.
Molday **ION**™, MION, USPIO, SPIO, MRI, Prussian blue, spermidine, nanoparticle, iron oxide, colloid, cell labeling, receptor mediated endocytosis, conjugation, polyamine, zeta potential, BioPAL, Brookhaven Instruments Corporation

Summary. Application Note 5 demonstrates a simple method for conjugating amine-containing ligands to **CL-30Q02-6C** using standard bio-conjugation techniques. Using the chemistry outlined in this note, it should be possible to conjugate low molecular weight ligands onto **CL-30Q02-6C** or its fluorescent analog, **CL-30Q02-6C-50**. The only requirement is that the ligand needs a terminal reactive amine for conjugation. Cell labeling of NIH 3T3 cells using the spermidine conjugate of **CL-30Q02-6C**, a USPIO, is demonstrated.

Introduction.

The biological importance and biochemical pathways of the polyamines (putrescine, spermidine and spermine) are well documented(1). Eukaryotic cells internalize extracellular polyamines as well as synthesize them. Cellular uptake of polyamines appears to be universal in normal and cancerous cells in all species studied(2-5). Beyond mammalian cells, polyamine transport has been documented in insect cells(6). Taking into consideration the information on polyamine transport, materials can be engineered that exploit this pathway to bring exogenous materials into cells.

Labeling cells with USPIO nanoparticles is a useful technology for visualizing cells with MRI. However, USPIO nanoparticles are poorly internalized by cells without the use of transfection reagents. Some success in cell labeling has been reported with SPIO nanoparticles such as Feridex® in conjunction with a transfection agent such as protamine sulfate(7,8). Other attempts have used polyanionic surfactants with polycationic transfection agents such as poly-l-lysine or Lipofectamine™ (9,10). These attempts were modestly successful, but required exact mixtures, lengthy preparation time, and the supply of the now discontinued commercial SPIO, Feridex.

Using the polyamine transport system to load cells with drug-polyamine conjugates is not a new concept. For instance, visualization of the polyamine transport used a spermidine fluorescent conjugate(11). In this application note we (i) present a method for the synthesis of a spermidine-USPIO conjugate, (ii) suggest a general method for conjugation of other amine containing ligands to **CL-30Q02-6C** and (iii) demonstrate the efficient labeling of NIH-3T3 cells using **CL-30Q02-71** without the use of transfection reagents.

Materials and Methods.

CL-30Q02-6C [5mg Fe/ml]	BioPAL
CL-01-50 Prussian Blue Reagent Pack	BioPAL
CL-01-52 25% Glutaraldehyde	BioPAL
CL-01-53 40% Formalin	BioPAL
CL-01-51 PBS++	BioPAL
0.2 M MES buffer, pH 6	BioPAL
0.1 M MES buffer, pH 6	BioPAL
Spermidine trichloride	MP Biomedicals
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDAC)	TCI
N-Hydroxysulfosuccinimide (sulfo-NHS)	Fisher Scientific (Pierce)
100kDa MWCO Polyethersulfone membrane	Millipore cat#: PBHK02510

Synthesis

Conjugation of spermidine to **CL-30Q02-6C** was accomplished using EDAC and sulfo-NHS. The synthesis was performed in five steps at a scale of 10mg Fe.

1.) Add 2ml of **CL-30Q02-6C** [5mg Fe/ml] [$2.1x10^{-9}$ moles of particles] to 2ml 0.2M MES (pH 6.0) all contained in a 10ml round bottom flask. Place a small magnetic stir bar into the round bottom flask and clamp above a magnetic stirrer, stir at a medium setting.

2.) Dissolve 54mg of spermidine trichloride in 2ml 0.1M MES (pH 6.0) in a 12x100mm glass test tube. Set aside until step 4.

3.) Dissolve 10mg of EDAC and then 12mg sulfo-NHS into the stirring mixture from step 1. React for 15 minutes at room temperature.

4.) Once the 15 minutes has expired from step 3, add the 2 ml of spermidine from step 2. React for 12 hours at room temperature.

5.) Ultra-filter the material from step 4 through five cycles against a 100kDa membrane with 200ml of distilled water for each cycle. The filtration process can be monitored by absorbance at 260nm, the λ_{MAX} for the sulfo-NHS. The product was brought to a final concentration of 1mg Fe/ml, adjusted to pH 7.4 using 1M HCl, and passed through a 0.2 micron filter.

The product, Molday ION Spermidine, (Catalog **CL-30Q01-71)** can be purchased from BioPAL.

Instrumental

Colloids (**CL-30Q02-6C, CL-30Q02-71, and CL-30Q02-6C-2A)** were sized by photon correlation spectroscopy using a 90 *Plus* particle size analyzer (Brookhaven Instruments Corp.). Samples were diluted to 1mg Fe/mL in saline, passed through a 0.1μm filter, and sonicated for one minute in a water-bath sonicator. A count rate between 50,000 and 500,000 counts per second was obtained. Data were collected for 4 cycles of 15 min and the cumulative results combined. Particle size was calculated by assuming refractive indexes of 1 and 0 for the real and imaginary components, respectively. Typical reproducibility for intra- and interassay variability yielded coefficients of variation of less than 6% and 9%, respectively. Nanoparticle sizes were calculated using an intensity-weighted log normal distribution. **Zeta Potential.** Zeta potential measurements for representative colloids were determined using a 90 *Plus* particle size analyzer (Brookhaven Instruments Corp.) with zeta potential modification. Samples were diluted to a concentration of 1mg Fe/mL (total metals) in 1mM KCl, passed through a 0.1μm filter, and sonicated for one minute in a water-bath sonicator. Samples were run using the following instrument parameters: Sample count rate, ~700 kcps; sampling time, 1 minute; conductance, ~400μs; current, ~1.05mA, electric field ~7.5V/cm. Data is reported as the mean zeta potential (mV) of ten measurements.

Cell Labeling

NIH-3T3 cells were grown to 70% confluence in DMEM with 10% BCS and 1X AAS in a 24-well plate at 37°C and 5% CO_2. The medium was aspirated and replaced with 37°C serum free DMEM media, supplemented with either **CL-30Q02-6C**, **CL-30Q02-71**, **CL-30Q02-6C-2A**, or **Control** (see Table 1). All test compounds were brought to a concentration of 100ug Fe/ml. The cells were incubated 18 hours. The cells were then washed with 37°C PBS++ and fixed with a 2% formalin/2.5% glutaraldehyde solution in PBS++ for 10 minutes. The cells were washed again with PBS++ and stained using the Prussian Blue Reagent set (**CL-01-50)**. Procedures for cell fixation and Prussian blue staining can be found on BioPAL's website

Results and Discussion.

Synthesis, chemical and physical properties. The outlined synthetic procedure involves minimal reagent preparation, can be done in an afternoon, is highly reproducible, and should work across a wide array of low molecular weight amine containing ligands. The covalent incorporation of spermidine to the starting

nanocolloid, **CL-30Q02-6C**, was supported by several simple analytical procedures. The product was ninhydrin positive while the starting colloid was ninhydrin negative. Passage of the product over G-25 columns equilibrated with 1M sodium chloride or 0.1M sodium hydroxide did not release ninhydrin positive material to the included volume and the product eluting in the void volume remained ninhydrin positive.

Figure 1. Zeta potential data obtained for **CL-30Q02-6C** (Panel A), **CL-30Q01-71** (Panel B), and **CL-30Q02-6C-2A** (Panel C). **CL-30Q02-6C-2A** was synthesized from **CL-30Q02-6C** using an identical procedure to that described for conjugation of spermidine except that spermidine was replaced by ethylene diamine. The blue and red lines represent theoretical and experimental results, respectively.

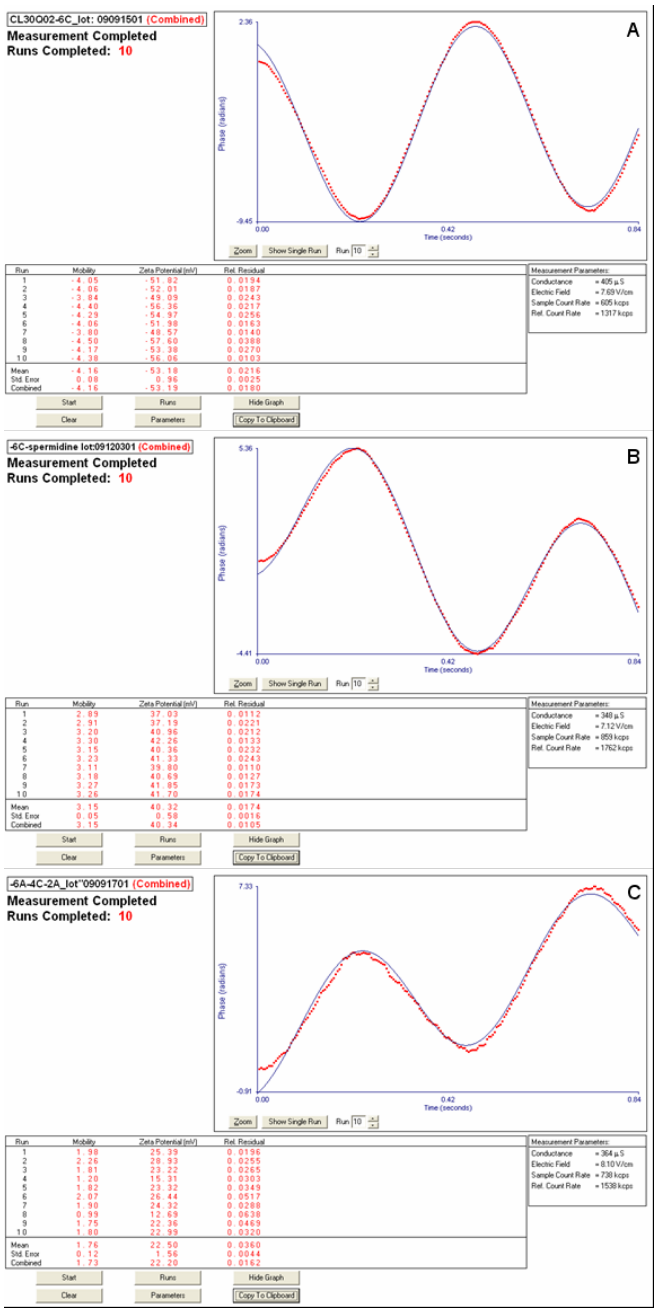

Figure 2. Images were taken at 400X magnification on a Nikon TE-2000-S microscope using bright field optics. Panel A are 3T3 cells treated with **CL- 30Q02-71** (spermidine USPIO); Panel B are cells treated with **CL-30Q02-6C-2A** (ethylene diamine USPIO); and Panel C are cells treated with **Control** (passively coated **CL-30Q02-6C** with spermidine). Cells were fixed and developed using Prussian blue staining to visualize the presence of USPIO iron. Bars represent 20 microns.

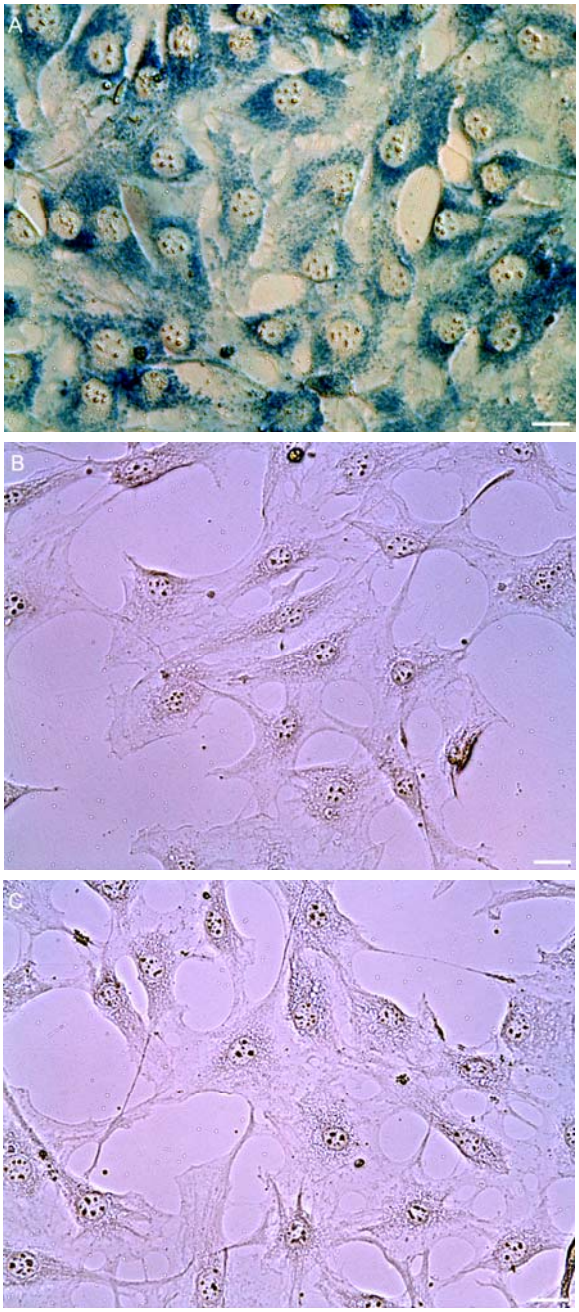

Table 1. Comparison of size, zeta potential and cell labeling for four USPIO nanocolloids. Size, zeta potential and cell labeling were performed as described in Methods.

Compound Number	Compound Name	Effective diameter (nm)	Zeta potential (mV)	Cell labeling
I	**CL-30Q02-6C**	**37.3**	**-53.2**	**No**
II	**CL-30Q02-71**	**35.8**	**40.3**	**Yes**
III	**CL-30Q02-6C-2A***	**40.2**	**22.5**	**No**
IV	**Control***	**38.8**	**-30.8**	**No**

***CL-30Q02-6C-2A** was prepared by replacing spermidine with ethylene diamine. Control was prepared by mixing **CL-30Q02-6C** with spermidine followed by exhaustive ultrafiltration.

Consistent with these observations was the change in zeta potential from negative of the starting material, **CL-30Q02-6C**, to positive of the product, **CL-30Q02-71**, indicating a net change in charge with the incorporation of spermidine's amines on the product particle (Table 1 and Figure 1). The zeta potential of the carboxyl-containing starting material is highly negative and indicates a high number of carboxyl groups associated with the nanoparticle. Similarly, the zeta potential of the product is highly positive and indicates a high number of amine groups associated with the nanoparticle. The sizes of the starting and product nanoparticles were the same demonstrating that no crosslinking of particles occurs during the conjugation of spermidine (Table 1).

Two control syntheses were performed by substituting ethylene diamine for spermidine (**CL-30Q02-6C-2A**) or by omitting EDAC and sulfo-NHS (**Control**) in the standard synthesis presented in Methods. The zeta potential of **CL-30Q02-6C-2A** and **Control** are strongly positive and strongly negative, respectively. The zeta potential of **Control** suggests that spermidine does not stick tightly to **CL-30Q02-6C** lending additional support that spermidine in **CL-30Q02-71** is covalently bound to **CL-30Q02-6C**. These four particles were prepared to compare their cell uptake and internalization in 3T3 cells.

Cell Labeling. **CL-30Q02-71,** the product conjugate, had the anticipated property of cell labeling (Figure 2A). **CL-30Q02-71** was tested for cell uptake using NIH-3T3 cells. After 18 hours of incubation, the cells were fixed and stained with Prussian blue. The blue areas indicate where **CL-30Q02-71** localized. The blue color results from of the reaction of iron with potassium cyanoferrate, the Prussian blue dye. **CL-30Q02-71** appears to be contained within vesicles in the cytoplasmic region of the cell and does not localize to the nucleus. To add supporting evidence that the spermidine was specifically interacting with the cell, a colloid of similar size and charge was synthesized substituting ethylene diamine for spermidine. The colloid synthesized with ethylene diamine, **CL-30Q02-6C-2A**, yielded a product with a size and positive zeta potential similar to that of the spermidine colloid (Table 1); however, the ethylene diamine conjugated colloid did not label cells (Figure 2B). Similarly, **Control** did not label cells (Figure 2C). Furthermore, the starting material **CL-30Q02-6C** had previously been tested for uptake in cells and was found to be negative (data not shown).

A spermidine nanocolloid could be synthesized for live cell viewing by substituting **CL-30Q02-6C-50** for **CL-30Q02-6C** in the five step synthetic method above**. CL-30Q02-6C-50** is the rhodamine B analog of **CL-30Q02-6C**. Alternatively, a small number of amino groups of spermidine on **CL-30Q02-71** can be modified to introduce a fluorophore. Careful monitoring of conjugation with the fluorescent dye is needed to not over-modify **CL-30Q02-71** with too many fluorophores per particle. This could reduce cell uptake due to saturation of amines, increased cytotoxicity, or induce cell death upon application of light from phototoxic effects.

CL-30Q02-71 as well as USPIO custom conjugations are available from BioPAL. Please inquire.

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