

Application Note 9.

Title. Molday **ION** Rose Bengal, **CL-50Q02-6A-53**, a cell labeling photodynamic theranostic USPIO JRH/EVG

Keywords.

Molday **ION**TM, MION, USPIO, SPIO, MRI, Rose Bengal, theranostic, photodynamic therapy, apoptosis, necrosis, fluorescence, nanoparticle, iron oxide, colloid, cell labeling, BioPAL

Summary. Nanoparticles are increasingly being used to label cells for MRI imaging and tracking, for *in vivo* quantification, and as drug delivery vectors. Unfortunately, normal cellular uptake mechanisms are inadequate to load cells with nanoparticles at sufficient levels to be effective. We have prepared a non-transfection-based cell labeling iron-oxide nanoparticle conjugated with the fluorescent molecule Rose Bengal. This novel material allows for MRI imaging of labeled cells and introduces the ability to kill the labeled cells on demand. Labeled cells can be selectively terminated upon exposure to a specific wavelength of light, yet remain viable under normal conditions. When exposed to light, cells die by apoptosis or necrosis depending upon the time and intensity of exposure. Molday **ION** Rose Bengal is easily and rapidly (2-16 hours) taken up by a variety of cells. Labeling is uniform and efficient (5-30% of the label is incorporated into cells). Labeled cells exhibit no signs of toxicity until exposed to the proper wavelength of light. The nanoparticles concentrate inside endosomes and are not found in the endoplasmic reticulum, golgi apparatus, nucleus, or any other cellular organelle. The nanoparticles are sterile and ready to use. Cell labeling is simple, chemically safe, and typically requires no more than one hour of laboratory contact time.

Introduction.

MION and MION-like superparamagnetic iron oxide nanoparticles have been extraordinarily useful in a broad range of applications including: immunoassays [1,2], detection and separation of cells, viruses, hormones, oligonucleotides, DNA and proteins [3-8], cell tagging, tracking, and imaging [9], targeted drug delivery [10]; gene delivery and therapy [11]; targeted hyperthermic treatment of cancer tissue [12], detoxification of biological fluids [13], and as a human MRI contrast agent for detection of liver tumors [14]. MRI provides valuable real time cell tracking information in regenerative medicine [15-17]. Recent interest, accordingly, has focused on developing *ex vivo* cell labeling methods using MION and other ultrasmall superparamagnetic iron oxide nanoparticle contrast agents (USPIO) for analytic uses in development of cell therapy technology.

There have been numerous issues surrounding *ex vivo* cell labeling using nanoparticles [18]. Ideally, one wants a material that will be rapidly and completely taken up by the target cells in culture. Unfortunately dextran-coated nanoparticles such as MION are not readily transported into the cell by normal nonspecific cell uptake mechanisms. Typically, less than 1% of MION is internalized by cellular endocytosis [19-21]. This low level of incorporation of the MRI contrast agent MION stresses the already low sensitivity of MRI technology. Accordingly, researchers have conjugated a variety of agents to nanoparticles including MION to increase the percentage uptake of nanoparticles. These agents have not proven to have widespread applicability either due to the difficulty of preparing such conjugated nanoparticles or the difficulty of obtaining the targeting moieties [22]. Nevertheless, researchers have pursued a number of avenues to improve the ability of nanoparticles to label cells [23,24].

This application note presents a simple method for labeling cells using Molday **ION** Rose Bengal, **CL-50Q02-6A-53**, a MION-like iron oxide nanoparticle that labels cells with high efficiency thereby presenting a general solution to cell labeling with MRI contrast agents. Molday **ION** Rose Bengal is a superparamagnetic iron oxide nanoparticle covalently conjugated with the fluorescent dye Rose Bengal and is designed to label cells.

Rose Bengal is a photodynamic therapy agent in clinical trials for cancer treatment. When illuminated at 540nm Rose Bengal emits at 550-600nm and generates an oxygen singlet, a reactive oxygen species (ROS) that cause cell damage and induce apoptosis or necrosis depending on the intensity and duration of light exposure.

Molday **ION** Rose Bengal labels cells without the need for transfection agents and accumulates in cellular vesicles. Labeled cells remain viable until photoactivation. Molday **ION** Rose Bengal can be used for MRI cell tracking and induced apoptosis studies.

Materials and Methods.

CL-50Q02-6A-53 Molday ION Rose Bengal [2mg 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
Dulbecos Modified Eagles Medium (DMEM)	HyClone
Bovine Calf Serum (BCS)	Gibco
Antibiotic-Antimycotic Solution (AAS)	Calbiochem
24-well plate, cell culture treated	Corning

Cell Labeling

(1) Serum free labeling of cells. Cells were grown to 70% confluence in DMEM with 10% BCS and 1X AAS in a 24-well plate at 37°C and 5% CO₂. The medium was aspirated and replaced with 37°C serum-free DMEM media, supplemented with **CL-50Q02-6A-53** at a concentration of 25ug Fe/ml. The cells were incubated 18 hours. The solution was aspirated after incubation. The cells were washed with Phosphate Buffered Saline (PBS) supplemented with Calcium and Magnesium and covered with complete media. The cells were visualized while living or fixed (2% formalin 2.5% glutaraldehyde) using an inverted fluorescent microscope (Nikon TE-2000).

(2) In an alternative procedure cells were labeled in media supplemented with serum. Cells were plated in a 24-well plate (1x10⁵ cells/well) in 0.5 ml DMEM containing 10% bovine calf serum (GIBCO) and incubated (humidified) at 37°C, 5% CO₂. After allowing the cells to adhere overnight, **CL-50Q02-6A-53** was added to the medium for 16 hours. The media was aspirated after incubation. The cells were washed with Phosphate Buffered Saline (PBS) supplemented with Calcium and Magnesium and covered with complete media. The cells were visualized while living (or fixed [2% formalin 2.5% glutaraldehyde]) using an inverted fluorescent microscope (Nikon TE-2000).

Instrumental

Colloids were sized by photon correlation spectroscopy using a 90 *Plus* particle size analyzer (Brookhaven Instruments Corp.). Zeta potential measurements were determined using a 90 *Plus* particle size analyzer (Brookhaven Instruments Corp.)

Microscopy

Microscopy, phase contrast and fluorescence, was performed using a Nikon TE-2000S instrument with a 20X Plan Fluor objective. A “dsRED” filter set was used for photoactivation and imaging of the Rose Bengal fluorescence. For the short pulse exposure of light, the light source was shuttered to 500ms; for long exposure 10 seconds, the shutter was left open until the all flurophore bleached. Images were captured using a Diagnostics Instruments 18.2 Color Mosaic CCD camera.

Results and Discussion.

Synthesis, chemical and physical properties. Molday **ION** Rose Bengal (**CL-50Q02-6A-53**) is a homogeneous, fluorescent iron oxide-based superparamagnetic (USPIO) contrast reagent designed to label cells efficiently and simply. **CL-50Q02-6A-53** can be visualized by both MRI and fluorescence. It is prepared from **CL-50Q02-6A** by reaction with N-hydroxysuccinimide ester of Rose Bengal. Rose Bengal is a fluorescent dye with an excitation and emission wavelength of 540 and 550-600nm, respectively. **CL-50Q02-6A-53** has an effective diameter of 35nm and a zeta potential of 31mV similar to **CL-50Q02-6A-50**.

How Rose Bengal's Photodynamic Action Works. Rose Bengal is a photodynamic therapy agent in clinical trials for cancer treatment (clinical trials.gov). When illuminated at 540nm Rose Bengal emits

at 550-600nm and generates an oxygen singlet, a reactive oxygen species (ROS) that causes cell damage and induces apoptosis or necrosis depending on the intensity and duration of light exposure.

Dose response of cell labeling. NIH-3T3 cells exhibit a direct relationship between the concentration of **CL-50Q02-6A-53** and cell uptake. The cells showed no signs of growth inhibition or toxicity from exposure to the reagent.

Cellular location of CL-50Q02-6A-53. Molday **ION** Rose Bengal isolates to endosomes, does not localize to the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, or any other cellular organelle, nor is it found on the surface of cells post labeling.

Retention of CL-50Q02-6A-53 within NIH-3T3 cells. Retention of **Molday ION** Rose Bengal in labeled NIH-3T3 cells is similar to that described for **Molday ION** Rhodamine B [see Application Note 3].

Viability of NIH-3T3 cells labeled with Molday ION Rose Bengal. Viability of NIH 3T3 cells labeled with **Molday ION** Rose Bengal when not exposed to light is similar to that described for **Molday ION** Rhodamine B [see Application Note 3].

Effect of light exposure upon cell death pathway. Photo-treatment of **Molday ION** Rose Bengal-labeled NIH-3T3 cells used a collimated and focused activating light source as a small circle thereby minimizing activation outside the field of view. The exposure time was either 500mseconds or 10seconds. With these exposure times cell death occurred exclusively within the exposed diameter and followed a morphology consistent with apoptosis (Figure 1) or necrosis (Figure 2) for the short and long exposures, respectively.

Figure 1C shows cells one hour after a 500 msecond light exposure. The rounding of the cells is suggestive of apoptosis. Figure 1E shows a selected and magnified field taken from Figure 1D. The rounded detached cells show the typical spheroid shape with associated blebbing lamellipodium consistent with apoptotic cell morphology. Inspection of Figure 1D illustrates the selective apoptosis of only cells exposed to the light source. Cells outside the area of light exposure remain viable.

Figure 2C shows **Molday ION** Rose Bengal labeled cells one hour after a 10 second light exposure resulting in the complete photo-bleaching of the Rose Bengal. Cells that were exposed to the center of the beam of light are necrotic while cells on the edges exposed to indirect light show a morphology consistent with apoptosis. Figures 2D and 2E show a cropped and magnified view of cells before exposure and one hour after a 10 second light exposure. The fields of view are taken from the center of images Figure 2A and 2C, respectively. Figure 2D shows living cells prior to the light exposure. Figure 2E shows necrosis as evidenced from the nuclear membrane detaching from the cell body. This is seen as a dark spherical nucleus with a phase light ring around the detached nucleus. The lamellipodium can also be seen to be detached, shriveled and blebbed.

The two modes of cell death correlate to length of light exposure and intensity of light. A short pulse of light generates ROS at sufficient levels to trigger the apoptosis signaling cascade. However, with a long exposure, the higher ROS generation, causes widespread cellular damage, completely destroying all cellular mechanisms.

Figure 1: Induced apoptosis of NIH-3T3 cells labeled with **Molday ION Rose Bengal** following pulsed light exposure. The montage below Panels A-C shows paired phase contrast and fluorescence images of NIH-3T3 cells labeled with **Molday ION Rose Bengal**. Panel A. 200X phase contrast image before photo-activation, Panel B. 200X fluorescence image at time of pulsed light exposure (500msecond exposure). The image is pseudo-colored to show fluorescent intensity. Panel C. 200X phase contrast image 1 hr after photo-activation. Panel D. 100X phase contrast of the same field of view as seen in Panels C to include a larger field of view. The red square indicates magnified view shown in Panel E. Panel E. Magnified phase contrast, cropped image from Panel D showing details of apoptotic cells.

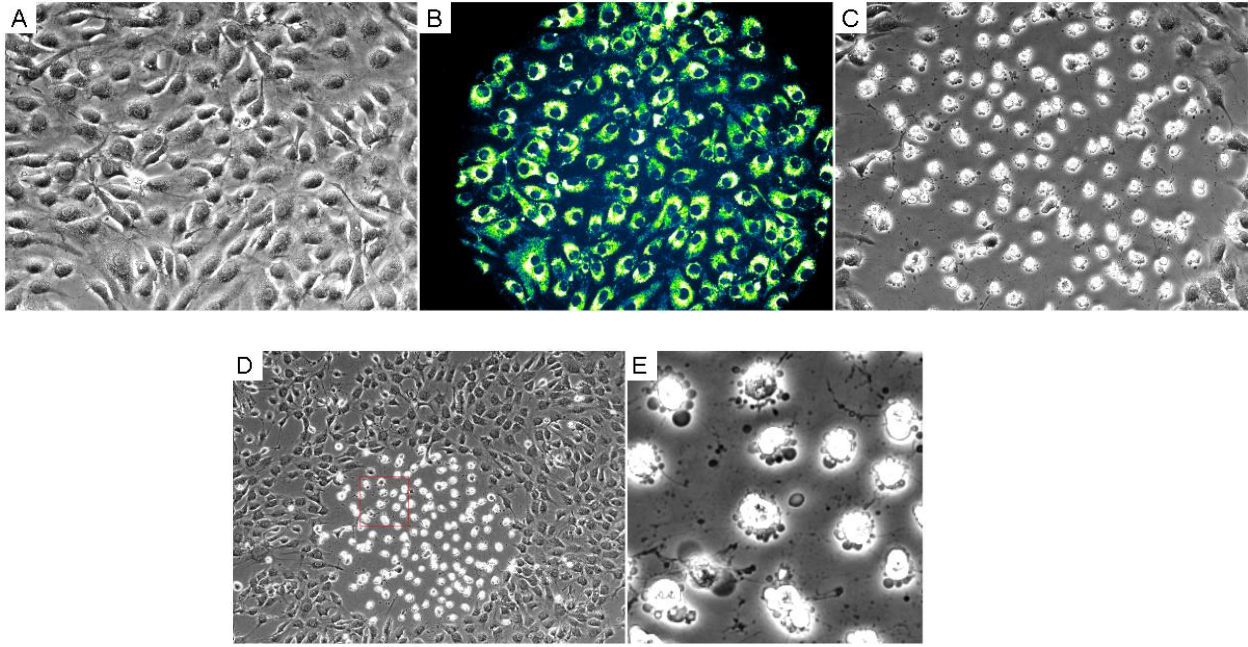
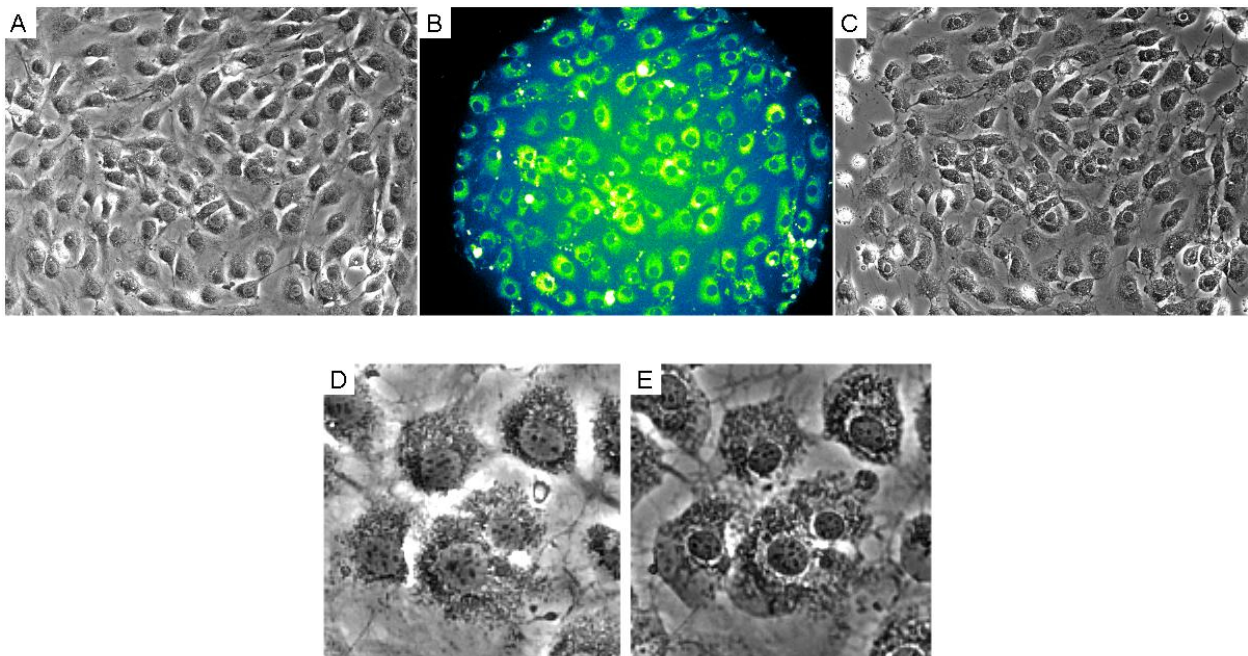


Figure 2: Induced necrosis following long light exposure of **Molday ION Rose Bengal**. The montage below shows paired phase contrast and fluorescence images of NIH-3T3 cells labeled with **Molday ION Rose Bengal**. Panel A. 200X phase contrast before photo-activation, Panel B. 200X fluorescence at time of activation (10 second exposure). The image is pseudo-colored to show fluorescent intensity. Panel C. 200X phase contrast 1 hr after photo-activation. Panel D. Magnified image taken from center of Panel A showing cells before exposure to light. Panel E. Magnified image taken from the center of Panel C one hour after exposure to light.



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