A method for measuring intracellular free magnesium concentration in platelets using flow cytometry

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Abstract. Magnesium is the fourth most abundant cation in the body and is involved in over 302 enzymatic reactions. Basic science research has implicated magnesium deficiency as a cause of insulin resistance which is related to hypertension, diabetes, hyperlipidemia and increased cardiovascular risk. Research in magnesium deficiency states has been hindered because magnesium is an intracellular ion and difficult to measure. Our goal was to develop a reproducible assay to measure intracellular magnesium in platelets. Healthy volunteers agreed to have blood drawn for magnesium measurement. Platelet rich plasma was harvested from a venipuncture specimen and run through the flow cytometer. A standard titer curve using known increasing concentrations of magnesium chloride was created for each specimen, and then with the other half the specimen was run to measure the true intracellular free magnesium concentration. 15 adults agreed to volunteer for this experiment. All standard titer curves for all specimens had a correlation of > 0.99. The mean concentration of intracellular free magnesium was 450.05 μM with a range of 203.68 μM to 673.50 μM. Intracellular free magnesium can reliably and reproducibly be measured in platelets using Mag Green fluorescent dye and flow cytometry. This should advance our ability to study magnesium deficient states.

Key words: magnesium, flow cytometry, measurement, platelet

The clinical significance of magnesium as an important intracellular cation has been implied for decades [1, 2]. Magnesium deficiency has been implicated in the pathogenesis of a host of clinical disorders [3-6] including insulin resistance [7, 8]. An editorial by Lawrence Resnick, M.D. in the March, 1997 issue of the American Journal of Hypertension states: “A link between magnesium, diabetes mellitus, and hypertension seems established beyond a reasonable doubt” [9].

Magnesium is the fourth most abundant cation in the body. It is involved over 300 enzymatic systems, such as adenosine triphosphate (ATP) metabolism, activation of creatinine kinase, adenylate cyclase, and sodium-potassium-ATPase. Magnesium deficiency has been implicated in such diseases as diabetes, hypertension, cardiac arrhythmias, acute myocardial infarctions, and atherosclerosis. It has come under increasing scrutiny in several publications [3, 4, 9, 10].

Although there is clear evidence that magnesium is an important cation in a number of diseases, there is a paucity of information on the exact pathophysiological role of magnesium because it is difficult to
accurately measure intracellular free magnesium levels, the biologically significant fraction. Only 0.3% of total body magnesium is present in the serum, which is the standard clinical test for magnesium homeostasis [11]. Much previous basic science and animal research measuring intracellular magnesium utilized nuclear magnetic resonance (NMR) measurements [12-14], ion-specific electrodes [15, 16], or fluorescence probes Mg-Fura 2 and a spectrophotometer [17-19]. We developed a reproducible assay using a flow cytometer and Magnesium Green™ (Molecular Probes, Invitrogen, Carlsbad, CA, USA) to measure intracellular magnesium in platelets [20, 21]. This assay should allow for wider measurement of magnesium for research and eventual clinical measurement.

The advantages of measuring magnesium in platelets versus erythrocytes or leukocytes relates to the fact that platelets are relatively easy to isolate and they are devoid of haemoglobin, which may interfere with fluorescence signals of magnesium-specific fluorescent probes. Moreover, regarding the vascular system, platelets have some features which resemble vascular cells, including their ability to contract and the expression of receptors to which vasoactive peptides bind [22, 23]. As such, platelets have been studied as surrogates of vascular cells [24]. Platelet magnesium levels represent the intracellular fraction, which is more important than plasma magnesium levels, because magnesium is essentially an intracellular cation with only a very small amount being present in the extracellular (plasma) milieu. It is thus evident that accurate measurement of platelet magnesium levels could provide important clinical information on body magnesium status in health and disease.

Materials and methods

Blood collection and platelet preparation

This study was approved by the Health Sciences Institutional Review Board (IRB) at the University at Buffalo. Blood samples were obtained in buffered Na Citrate tubes, using at least one tube per donor. Samples were either processed immediately or sat upright overnight at room temperature. Sample processing and all platelet manipulations were done on ice or at 4°C. Platelet rich plasma (PrP) was prepared by centrifuging the tube at 200 x g for 10 minutes, collecting the supernatant, and transferring it into a 15 mL polystyrene tube. Using a Coulter counter (Model Number Act10, Coulter Corp, Miami, FL, USA), the initial platelet count in the PrP was recorded. Next, 1.0 mL of the PrP was carefully transferred to a fresh 15 mL polystyrene tube and washed with supplemented Hanks containing Hank’s Balanced Salt Solution without Ca** and Mg**, 10 mM HEPES (Mediatech, Herndon, VA, USA), and Indomethacin (Indo, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) at a final concentration of 5 μg/mL. After centrifugation (1500 x g for 5 minutes) the pellet was gently resuspended in 1 mL of supplemented Hanks, counted and adjusted to a concentration of 20 x 10⁶ platelets/mL. This processing resulted in a considerable loss of platelets even with the addition of Indomethacin which helps inhibit platelet aggregation. Platelets, being sticky by nature, will adhere to the sample tube walls even in the presence of Indomethacin during the sample processing, which includes sample centrifugation steps. The number of centrifugations used in the procedure was minimized to help reduce platelet loss. Platelet recoveries ranged from 10 to 20 percent after the initial wash based on the pre and post counts using a Coulter counter.

Mag Green loading of platelets

Magnesium Green AM dye powder (Mag Green Invitrogen, Carlsbad, CA, USA) was resuspended with anhydrous dimethylsulfoxide (DMSO) to a final concentration of 5 mM. The DMSO/Mag green mixture was vortexed and then centrifuged at 15,000 x g for 1 minute. In a 1.6 mL microcentrifuge tube, 991 μL supplemented Hanks, 5 μl 20% Pluronic F-127 surfactant (Invitrogen, Carlsbad, CA, USA) diluted in DMSO, and 4 μL of the 5 mM Mag Green were combined. This 20 μM Mag Green stock was kept at room temperature and in the dark to avoid photo-bleaching.

In a clean 15 mL polystyrene tube, 750 μL of prepared platelets and 250 μL of the 20 μM Mag Green dye stock were combined and vortexed briefly. The sample was then placed in a 37°C water bath and incubated for 30 minutes. On entering the platelet, Mag Green dye is de-esterfied making it polar and unable to leave the cell. Mag Green-loaded platelets were then removed from the water bath, 500 μL of the platelets were diluted into 4.5 mL of freshly prepared supplemented Hanks to which 0.5 mM Na₂EDTA (Sigma-Aldrich) had been added, then gently mixed by inverting twice. The loaded platelets were centrifuged at 1500 x g for 5 minutes at 4°C. The supernatant was aspirated and 1 mL of supplemented Hanks was added using a 1 mL pipettor to gently break up the pellet. The volume was adjusted to 5 mL and the platelets were incubated at
37°C for an additional 30 minutes in supplemented Hanks to allow the platelets to process the internalized Mag Green. After this treatment, a sample of platelets was withdrawn to measure constitutive levels of Mg++. To the remaining platelets, which would be used for the standard curve, ionomycin (Iono, Sigma-Aldrich) at a final concentration of 1 μg/mL was added to permit the free transport of Mg++ ions through the cell membrane.

**Standard curve preparation and platelet assay**

A 1M MgCl₂ stock solution (Sigma-Aldrich) was prepared in ddH₂O and then further diluted to 64 mM. Two fold serial dilutions were prepared from the 64 mM stock (ranging from 32 to 1 mM). Each tube was stored at 4°C and wrapped with parafilm between uses to prevent evaporation. These standard curve stocks are 10x working concentrations that would subsequently be diluted.

While the platelets were being loaded with Mag Green, typically 7 polypropylene tubes (12x75 mm) were setup for the following conditions:

1. Constitutive Mg++ (25 μL supplemented Hanks only);
2. Baseline (25 μL supplemented Hanks only);
3. 100 μM MgCl₂;
4. 200 μM MgCl₂;
5. 400 μM MgCl₂;
6. 800 μM MgCl₂;
7. 1600 μM MgCl₂.

To tubes 1 (constitutive) and 2 (baseline) 25 μL of supplemented Hanks were added and to each of the

![Image](image_url)

**Figure 1.** Platelets processed for the determination of intracellular magnesium were acquired on a FacsAria (BD Biosciences). Dot plot A shows the FSC and SSC pattern with a singlet gated (R1) applied to the population. A sample of the platelets were stained with allophycocyanin (APC) tagged CD41a antibody which has a specificity for platelets. Histogram B shows the signal from the APC-CD41a stained platelets compared to the unstained. Histogram C shows the signal from platelets that have taken up and processed the Magnesium Green dye compared to the background signal without the dye.
standard curve tubes (3-7) 25 µL of the appropriate working stock was added. Next, 225 µL of Mag Green loaded platelets without ionomycin was added to tube 1 (constitutive) and 225 µL of Mag Green loaded, ionomycin treated platelets was added to each of the remaining tubes (2-7). All tubes were incubated at 37°C for at least 1 hour. Samples were chilled on ice for a minimum of 5 minutes before assaying on the flow cytometer.

Flow cytometric analysis

Samples were acquired on the FacsAria (Becton-Dickinson, San Jose, CA, USA) using a 488 nm laser source. The emission from the Mag Green was collected using a 530/30 band pass filter. A minimum of 20,000 events gated on Forward Scatter (FSC) versus Side Scatter (SSC) to exclude doublets and debris were collected. The 530/30 channel voltage was established using unstained platelets to set the baseline and the brightest positive stained platelets were evaluated to ensure they would be on scale. Brightly stained platelets fell near the beginning of the fourth decade on a 4 decade log scale. Data was analyzed using WinList Flow Cytometry analysis software (Verity Software House, Topsham, ME, USA).

Results

The primary gate for the population was forward light scatter (FSC) and side light scatter (SSC), having both set in a linear mode (figure 1A). To confirm that these events were indeed the platelets, a sample

![Figure 2](image-url)

Figure 2. A donor platelet sample was processed for intracellular magnesium level detection for this figure. The titer curve was created by treating platelet samples with Ionomycin and then adding increasing concentrations of MgCl₂. The range for the titer curve was from 25 µM to 1600 µM. The linear portion of the curve, 200 µM to 1600 µM was used to determine the regression curve fit. The Ionomycin, no Mg²⁺ point represents the sample that was treated with Ionomycin in the absence of external magnesium. The test sample point is platelets not treated with Ionomycin which should give the constitutive intracellular level of Mg²⁺. The intracellular Mg²⁺ level was determined to be 479 µM for the test sample.
was stained with allophycyanin (APC) tagged antibodies and data was collected on the FacsAria as excited by a 633 nM laser and collected through a 660/20 band pass filter. The results in figure 1B show that the cells are CD41a positive and backgating analysis (not shown) using the FSC/SSC window confirm that the CD41a positive population is in fact the same population used for Magnesium Green analysis. A representative histogram of Magnesium Green stain platelets is seen in figure 1C. The channel shift between the lowest concentration of Mg++ in the titer curve (100 µM) to the highest concentration (1600 µM) averages about 400-500 in mean fluorescence intensity (MFI). The median of the positive peak was used to determine the MFI of the positive signal.

Figure 2 shows a titer curve developed using the donor platelets. The range for this curve was expanded to show the sensitivity of the assay. The magnesium titer curve range (solid line) is from 25 µM Mg++ to 1600 µM Mg++. Values occurring on the magnesium titer curve that fall below 200 µM are no longer in the linear range and the curve eventually plateaus at 50 µM and below. This inflection point is at the level where the platelets treated with Ionomycin, but no external Mg++ fall. The linear portion of the curve used for determining the titer curve is from 200 µM to 1600 µM for sample analysis. The regression curve analysis of the linear portion consistently gave R values near or above 0.99. The constitutive level of Mg++ (479.22 µM) in the test sample was established by applying the recorded MFI for that sample to the titer curve regression formula and calculating the value. The no Mg++ (fill triangle) represents the control sample that was treated with the ionophore, Ionomycin, in the absence of endogenous magnesium.

Figure 3 shows an intra-assay test done on a single individual. The platelet rich plasma (PrP) was divided into three samples (A, B and C) before processing for intracellular magnesium. All other conditions of the assay were the same, including the source of the buffers and dye. The results show a close correlation between the processed samples according to their resulting titer curves and the constitutive levels of Mg++ 285.86 (C, open square), 299.70 (A, filled diamond) and 296.01 µM (B, open triangle), respectively seen in each sample. Because of the daily variations of intracellular magnesium levels within the same donors, intra-assay results for the individual donor will vary. A typical example is from the donor cited in figure 3, where intracellular...
Mg++ levels over four different days measured 251.24, 470.22, 293.85, and 429.44 μM. The first three data points were measured over three weeks at one week intervals and the last data point was measured three months later.

Figure 4 demonstrates the need to apply separate titer curves for each donor using their own platelets to develop the curve. In this example three donors were assayed for internal Mg++ levels. As seen, the titer curves do not match between the samples, although the curves for individuals A and C coincide much better than B. The magnesium level calculated for each donor was A, B and C. In most studies, different donors processed on the same day using identical sources for the buffers and Magnesium Green dye gave varying levels of MFI, resulting in titer curves having MFIs that did not overlap.

Fifteen individual donors were assayed for intracellular magnesium using the protocol. The data was collected over a period of 3 months. The results are seen in figure 5. The mean of these is 450.05 μM Mg++. The donors were measured on different days and the Mg++ levels were measured using their unique titer curves. There is a range of 203.68 μM to 673.50 μM Mg++ in this set with a definite clustering of data points around 450 μM. The main clustering of data points fell in the range of 350 to 550 μM.

Discussion

The present paper describes a novel method using Mag Green and flow cytometry to measure intracellular free magnesium concentration. Using the described method, we successfully measured intracellular free magnesium levels in platelets from healthy volunteers. The method is reliable, sensitive and accurate. This method has several advantages over previously described methods of measuring intracellular free magnesium in that it is less expensive than nuclear magnetic resonance and can poen-
tially be used in any commercial laboratory that has a flow cytometer. This is the first method that we have found which uses a fluorescent dye and that creates an individual titer curve for each sample, thereby increasing the reliability of the result. All of the titer curves had very high R values of 0.99 or greater. Using a simple, rapid flow cytometric approach to measure Mg++ level, we obtained a mean of 450 μM ± 137 μM (range 204-674 μM) in 15 healthy donors. This compares favorably to the study of Takaya et al. [25] who measured Mg++ levels in platelets from 45 healthy donors using mag-fura-2 and fluorescence spectrophotometry and obtained an average value of 468 ± 132 μM. Using platelets and a similar assay, Hiraga et al. [26] reported a slightly lower Mg++ range (353 ± 85 μM) in one study with 30 controls, but in a parallel study using the same method this group obtained a normal range of 460 ± 140 μM (n = 24 donors) [27].

It was noted that there was an interpersonal variation in magnesium levels of up to 50% in individuals. Not having a medical history of the volunteers, it is hard to speculate on why this occurred. One of the volunteers was known to have taken magnesium supplements on an intermittent basis. Also, magnesium levels are related to magnesium depletion states, chronobiology, and light sensitization. This may have affected some of these levels [28-30].

Further work needs to be performed to establish a normal intracellular free magnesium range using this flow cytometric approach before this procedure can be useful as a clinical test. However, for now, our method can be utilized for research purposes where the measurement of intracellular magnesium is important.

Conclusion

Measuring intracellular magnesium in platelets using magnesium green dye and flow cytometry can be reliably accomplished. This can enhance our ability to conduct clinical research on magnesium.

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References
