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Journal of Photochemistry and Photobiology A: Chemistry 189 (2007) 147–152
Journal of Photochemistry and Photobiology A:Chemistry
www.elsevier.com/locate/jphotochem

Poly(methacrylic acid) enhances emission of dye stain extracted from bacterial spores: Fluorimetric quantitation in aqueous samples

Guilford Jones II, Pavel Landsman *

Photonics Center, Boston University, Boston, MA 02215, United States

Received 28 November 2006; received in revised form 11 January 2007; accepted 20 January 2007
Available online 26 January 2007

Abstract

A new fluorometric procedure is demonstrated for fast detection of dormant bacterial endospores in suspension. The method is based on quantitative uptake of the cationic dye stain malachite green (MG) by permeabilized spores. Specifically bound stain is extracted from the spores into poly(methacrylic acid) (PMAA) acidic solution. The steady-state fluorescence enhancement of MG in buffered PMAA-citrate is then employed for determination of dye uptake by spores at the nanogram scale. The assay tolerates the presence of anions, surfactants and other potentially masking species, that may be present in environmental samples. The analytical procedure is simple and allows sensitive ($<10^5$ ml^{-1}) identification of bacterial spores in aqueous samples within 1–2 h, in presence of possible masking factors, such as salts (buffers), surfactants and neutral particles.

Keywords: Fluorescence enhancement; PMAA; Malachite green; Bacterial spore detection; Fluorescent assay

1. Introduction

Bioanalytical assays allowing fast identification and quantitation of bacterial spores are demanded in many fields, including biodefense-related applications [1]. Methods for spore identification based on microscopic or microanalytical instrumentation [2] or on strain-specific immunochemical [3] and DNA-targeting reagents [4] are not suitable for fast detection purposes. The biochemical marker most suited for fast spore detection is calcium dipicolinate, which allows discrimination of all known species of bacterial spores from other bioparticles by standard fluorimetric assay [5]. However quantitative extraction of dipicolinate from spores require multi-step protocols, whereas its assay in solution is sensitive to ubiquitous environmental factors, e.g., phosphate ions [6,7].

Spectroscopic measurement of dye uptake has been broadly used for fast total count of surface-attached cells in various bioanalytical systems; e.g., in the sulforhodamine B (SRB), crystal violet (CV) and neutral red (NR) assays, the count is evaluated via UV–vis spectroscopic measurements of cell-extracted dyes [8–12]. However the sensitivity of UV–vis dye uptake assays is limited ($\sim 10^5$ mammalian cells/ml), whereas more sensitive fluorimetric modifications of these assays have not been developed. As yet, a dye uptake approach has not been suited for count of non-adherent (suspended) cells. Consequently, no methods based on direct spectroscopic probing have been developed for quantitation of dormant bacterial spores that are non-adherent to surfaces and virtually impermeable for known histological stains.

Triarylmethane (TAM) dye stains, including malachite green (MG) (Fig. 1, left) have long been applied in microbiology for Gram-differentiation of bacterial species. Malachite green, in combination with special hot-acid permeabilization procedure, has been recently applied for bacterial spore staining [13]. Staining potency of the TAM dyes toward spore-forming bacteria is very high (50–100% uptake by weight, in case of permeabilized *Bacillus* spores [14]). This feature has not been so far fully employed in fast methods for biodetection of microbial species. Among the TAM stains, only crystal violet has been used in dye uptake assays, for UV–vis evaluation of bacterial biofilms adhered to plastic surfaces [11].

The disadvantage of the TAM dyes related to analytical microbiology is that they are essentially non-fluorescent in aqueous solution [15,16]. Recent reports [17,18] indicate that certain polyelectrolytes, particularly polymethacrylic acid (PMAA), entrap these dyes within hypercoiled clusters formed

* Corresponding author. Tel.: +1 617 353 8655.
E-mail address: landsmap@bu.edu (P. Landsman).

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doi:10.1016/j.jphotochem.2007.01.024

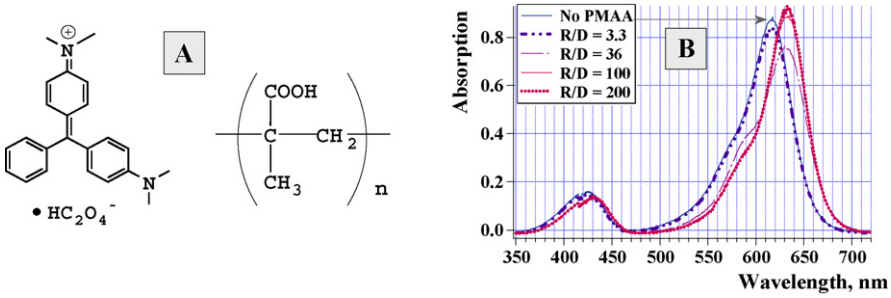


Fig. 1. (A) Structures of malachite green (left) and poly(methacrylic) acid (right); (B) PMAA-mediated changes (batochromic shift) observed in the UV–vis spectra of malachite green. *Conditions*: 5 mM citrate pH 4.0; [MG] = 15 μM, PMAA amounts vary from 45 μM to 3 mM. Corresponding polymer residue/dye (R/D) concentration molar ratios are shown in the inset.

by polymer chains at lower pH [19]. PMAA hypercoils provide molecular environment of high microviscosity for the dye molecules and dramatically increase life times and quantum yields of the studied TAM dyes. The current work introduces a dye uptake and elution protocol for fluorescent quantitation of bacterial spores in suspension. It employs PMAA-mediated enhancement for sensitive fluorometric determination of eluted dye in solution. Simultaneously PMAA serves as extracting agent ensuring quantitative recovery of spore-bound dye.

2. Experimental

2.1. Materials and measurements

Malachite green (MG), as 97% pure oxalate salt and dodecylamine (DodA) were obtained from Aldrich and used as received. Molecular weight of MG was calculated as that of single fluorophore molecule (3/2 oxalate salt, i.e. 463.55 Da). Polymethacrylic acid (PMAA) with average molecular weight 100 kDa was purchased from PolySciences Inc. Tween 20 (polyoxyethylene (20) sorbitan monolaurate, OmniPur grade) was from EM Science. All other reagents, of spectroscopic or analytical grade, were from VWR. All aqueous solutions were prepared in Millipore H_2O and filtered through 0.2 μm polycarbonate syringe filters (Corning) Absorption spectra were recorded on a DU-640B Beckman spectrophotometer. Steady state emission spectra were recorded on Felix/Timemaster fluorometer (Photon Technology International Inc.), using a slit width of 10 nm.

All measurements were performed at ambient temperature (~22 °C), in polystyrene two-sided and four-sided cuvets with 1 cm optical path (VWR). Microplate reader fluorescence measurements were performed in triplicates in black 96-well plates (Greiner), using SpectraMax Gemini XS fluorescence plate reader (Molecular Devices). Quantitations of measurements were based on relative emission intensities rather than absolute quantum yields. For all the samples used in fluorescent quantitations, the MG molar extinction at 610–650 nm was kept <0.02, in order to assess the possible effects of light re-absorption by the dye solutions. The pH of the aqueous solutions was monitored using an Orion 420A pH meter. Calculations were performed using Igor software v.4.0.5.1 (Wavemetrics).

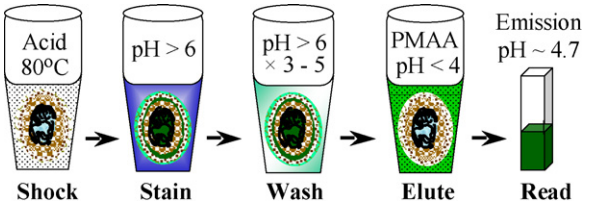
2.2. MG partitioning to the PMAA microphase

Partition coefficients (phase equilibrium constants), K_p, for distribution of MG between an aqueous phase and an aqueous PMAA environment, were calculated by the Benesi–Hildebrand method [20], from PMAA titration experiments at fixed MG concentration. The equilibrium was assumed to invoke PMAA environment for MG as thermodynamically homogeneous polymer phase. The apparent phase equilibrium constant K_p between PMAA (fluorescent) and aqueous (essentially non-fluorescent) phases was estimated as $K_p = MG_p/(MG_w P)$, where MG_p and MG_w are the mole fractions of MG in the polymer and water phases, respectively, P the varied polymer (per residue) concentration, and I-emission enhancement (assumed linear with MG_p–($K_p P$)). The value of K_p was then evaluated from the slope of the linear form $1/I = (1/P)(K_p/I_{max}) + 1/I_{max}$.

2.3. Spore cultures and samples for analytical procedures

Bacterial endospore cultures (*Bacillus subtilis var.* "*niger*") were obtained from US Army Research Laboratory facility, as desiccated powders. Powdered cultures were suspended and stored at 2 °C in double distilled water (DDW) or 40% ethanol—DDW. They contained ~10^8 to 10^9 spores per mg of dry weight, as estimated from the direct counts of the samples. The homogeneity and quality of the spore cultures and stock suspensions were examined microscopically (AccuScope 3016 model equipped with phase contrast objectives), at magnification 400× in water suspensions. All spore stocks used in the assays appeared as homogeneous suspensions of >90% phase-bright (non-germinated) spores, dia 0.8–1.2 μm. Before the "acid-hot" pretreatment, the spores demonstrated only minor staining when exposed to the malachite green staining solutions.

Counts of spore stocks were performed in multiplicate samples of the non-stained spore stock suspensions, diluted in the range ~10^7 to 10^9 spores/ml, using Petroff–Hausser counting chamber (VWR) in the phase-contrast field, at magnification 400×. For more accurate counts, 0.1% (final concentration, v/v) of nonionic surfactant Tween 20 was added to the counted samples, in order to suppress the formation of aggregates. Prior to the assay, the suspended cultures were washed with plenty of ice-cold DDW and then were kept on ice until measurements (Scheme 1; Section 2.4). Powdered microparticles



Scheme 1. A protocol for determination of bacterial spores by PMAA–MG elution assay.

(polystyrene—2% cross-linked divinylbenzene, PSDVB, dia. 2–8 μm; silica powders of various microparticle sizes; Aldrich) used in the control experiments were washed and treated in the same way as spore cultures.

2.4. Malachite green uptake assays with bacterial endospores

The dye uptake assay protocol (Scheme 1) was repeatedly applied under identical conditions, to the samples containing spores of *B. subtilis var.* "*niger*", also known as *B. atrophaeus* and *B. globigii* [2,5]. Triplicate spore samples were permeabilized according to the known procedure [13,14] ([25 mM] oxalic acid, 5 min at 80 °C, sonication 15 s) and then neutralized with sodium citrate to final pH 6.0, at ambient temperature. Average cell losses during the assay were estimated in separate counts of the accordingly pre-treated and stained spores, under the assay conditions. They remained within the range ~10–40% of the initial count, i.e., within the reported accuracy of the assay.

For the MG uptake measurements (Scheme 1), the dye was added to the permeabilized spores to the 0.1 mM final concentration. The samples were sonicated 15 s and rotated (240 rpm) for 30 min at ambient temperature. Each sample was then mixed with 20–50 μg/ml PSDVB particles (final suspension) and supplied with 0.05% SDS (final concentration). (Addition of PSDVB particles and SDS was found to improve consistent recovery of the spores during the washing cycles. Control samples for the dye background estimation contained the same amounts of PSDVB particles, with no spores.) The suspensions were taken to clean assay tubes and quickly washed 3× with washing buffer (10 mM citrate, pH 6.0 with 0.05% SDS) and 2× 10 mM SDS-free citrate (by centrifugation, 7 min at 7000 rpm, or 5200 × *g*).

The washed spore pellets were resuspended in the aliquots of elution buffer (50 mM citric acid, 3 mM EDTA, 50 mM_R PMAA; final concentrations). The samples were incubated 20 min in 80 °C water bath with vigorous agitation and sonication. After the final spin (10 min at 10,000 rpm, or 10,600 × *g*), the collected supernatants were adjusted to pH 4.5–4.9 with sodium citrate. Their spectroscopic readings (UV–vis absorbances at 615 or 630 nm; 620:650 and 428:650 nm fluorescence intensities in case of single cuvet fluorimetry; 428:655 nm fluorescence with filter cutoff 630 nm, in case of plate reader measurements) were corrected for the background of the corresponding spore-free samples. The absolute amount of spore-bound dye eluted into PMAA solution was determined using standard MG-PMAA calibration plots obtained under same conditions (Figs. 3C and 5).

3. Results

3.1. PMAA-mediated alterations in the UV–vis and fluorescent spectra of malachite green

Except where noted, interactions of malachite green to PMAA were studied at acidic pH, where the compact form of the (largely unionized) polyelectrolyte is dominant [19]. The metachromasia observed in the UV–vis spectra on addition of PMAA to aqueous solutions of MG includes a significant bathochromic shift (616 → 632 nm) at fixed dye concentration (i.e. at growing PMAA residue-to-dye (R/D) molar ratio) (Fig. 1B). Similarly to other reported PMAA–dye interactions, these changes accompany formation of MG contacts with lipophilic and hydrophilic/charged moieties [15,17,21,22]. In the absence of PMAA, in 1–50 mM aqueous citrate (range of pH 2.5–6.0), MG at dye concentrations up to 5×10^{-6} M showed essentially no fluorescence above background, at excitation wavelengths ranging from 350 to 700 nm. In aqueous PMAA citrate-buffered solutions (range of pH 2.5–5.5) enhancement of fluorescence could be observed for MG, starting at dye concentrations as low as [MG] ≤ 10^{-8} M. The samples produced

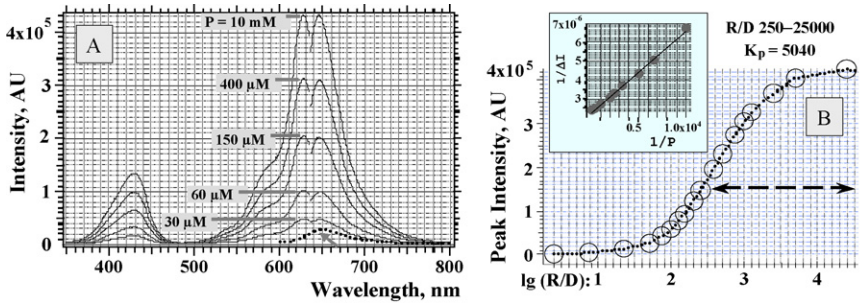


Fig. 2. Fluorescence spectra from titration series malachite green (fixed concentration [400 nM] in 20 mM citrate, pH 4.0)–PMAA (*P*, polymer concentration per residue, varied from [30 μM] to [10 mM]; R/D, residue-to-dye molar ratio, changed from 75 to 25,000, correspondingly). (A) Absorption (at emission wavelength 653 nm, on the left) and emission (excited at 622 nm, on the right) spectra. Emission spectrum at excitation wavelength 427 nm (*P* = 50 μM, dotted line at the bottom) is indicated with arrow. (B) Intensities of emission maxima (excited at 622 nm) at peak wavelength (~646 nm), plotted vs. R/D ratio. Inset: Benesi–Hildebrand linearization of the binding data at increased amounts of PMAA (R/D range indicated with double arrow), with constant K_p of dye partition between polymer and water phases obtained from the slope.

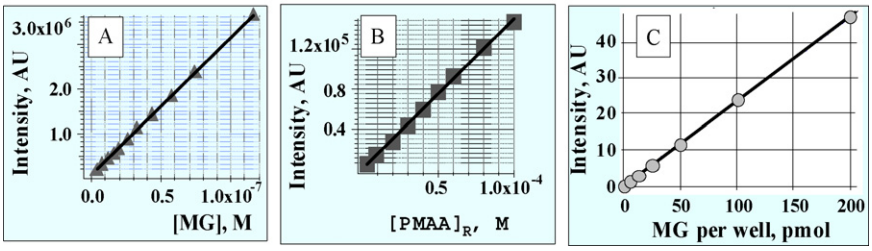


Fig. 3. PMAA–malachite green (MG) fluorescence enhancement. (A and B) Linear concentration plots of peak emission intensities at 626:645 nm vs. varied concentrations of components, from typical titration experiments. (A) Fixed polymer concentration $[PMAA]_R = 0.5\,mM_R$ (per residue), dye titration from 5 to 120 nM, in 5 mM citrate, pH 4.0; slope $3.07 \pm 0.01 \times 10^{13}\,M^{-1}$. (B) Fixed dye concentration 400 nM, polymer titration from 5 to 100 μM_R in 20 mM citrate, pH 4.; slope $1.53 \pm 0.03 \times 10^{9}\,M_R{}^{-1}$. (C) Malachite green quantitation in plate reader mode. Emission intensities at 430:650 nm and filter cutoff 630 nm were calculated in triplicates, with dye-free control triplicates subtracted.

a single emission maximum at long wavelength (642–645 nm) with the wavelength position not dependent on excitation wavelength (Fig. 2A). Comparison with emission spectra of the previously characterized complexes of other triarylmethane dyes with PMAA in hypercoil conformation [17] leads to an approximate absolute quantum yield for MG–PMAA of ~0.01.

3.2. Binding saturation of MG–PMAA for hypercoiled polymer

The saturation point at which the effect of added polyelectrolyte reaches a maximum in fluorescence enhancement is shown in Fig. 2B (R/D = ca. 3000). The apparent MG partition coefficient (K_p) between polymer and water phase was rather high ($K_p \sim 5000$) at polymer amounts R/D > 250 (estimated by the Benesi–Hildebrand method at fixed dye concentration; Fig. 2B, inset). The Benesi–Hildebrand treatment of the emission intensities and shifts of emission peaks versus dye concentrations, checked at various fixed concentrations of PMAA, also confirmed high affinity binding (10^6 to $10^8\,M^{-1}$) of MG in PMAA hypercoiled state, over the same R/D range (not shown). Overall, linearization of titration experiments confirm high affinity, homogeneity and conformational stability of the dye binding sites in the PMAA chains over this whole range, independently of the polymer concentration in solution.

3.3. Malachite green fluorescence enhancement in PMAA

The intensity of the 622:645 nm steady state emission signal was strong enough to quantitate MG solutions at a concentration level of a few nanomolar for MG–PMAA samples. At [PMAA] = P = 0.5 mM per residue, in 5 mM citrate pH 4.0, the emission 622:646 nm is linear in the range of MG concentrations 5×10^{-9} to 1×10^{-7} M (Fig. 3A). This linearity enables accurate fluorometric quantitation of MG concentration in analytical samples for which the UV–vis OD range at 616 nm (major MG absorbance peak) is 0.001–0.01 AU (i.e., amounts undetectable spectrophotometrically). At fixed MG concentration 400 nM, the emission also linearly depended on PMAA concentration in the range 5–100 μM_R (per residue concentration) (Fig. 3B).

In the fluorescent plate reader mode, the alternative excitation wavelength 430 nm (associated with the $S_2 \rightarrow S_0$ transition of MG [15]) was especially convenient for measurements, providing relatively large (220 nm) shift in emission and consequently improved signal-to-background ratio for higher dye dilutions. There, the effect of PMAA complexation enables detection of as little as few picomoles MG per well of a 96-well plate (Fig. 3C). In general, the use of the PMAA host polymer enabled more than a 100-fold gain in analytical sensitivity for MG detection, as compared to UV–vis-based spectroscopic methods (limited to 200–400 nM detection for typical instruments).

Surfactants and polyanions (e.g., phosphate, citrate, EDTA) are commonly employed in spore culturing and collection procedures and were also used herein in spore treatment and staining protocols. These compounds were therefore tested for their possible disruptive effect on the emission of MG–PMAA complexes [14,16]. For aqueous solutions of polymer $[PMAA]_R = 20$ mM and [MG] = 400 nM at constant pH (within the range 2.5–5.0), the presence of citrate and acetate up to 0.2 M, or EDTA and oxalate as additives, up to 0.1 M, did not affect the MG–PMAA UV–vis and emission spectra (not shown). Under the same conditions, for concentrations of the representative surfactants below 1.0 mM, only a weak effect on the intensity of emission of MG–PMAA was observed (Fig. 4B). This tolerance of MG–PMAA emission toward surfactants, despite variations in structure and charge, indicates a high stability for MG–PMAA complexes, apparently deeply buried within PMAA hypercoiled clusters. The emission signal remains essentially stable to pH variations within the range of pH 3.0–5.0, with the relative emission maximum attained at pH 4.5–4.7 (Fig. 4A).

3.4. Detection of bacterial endospores in suspension based on reversible uptake of malachite green

The optimized protocol (Section 2.4) included the following five steps (Scheme 1):

(1) permeabilization of dormant spores toward malachite green uptake, via heat-shock in the presence of oxalic acid, based on modified spore staining protocol [13,14];
(2) neutralization, with the resulting pH of the sample ~6. More alkaline pH, leading to formation and surface precipitation of the leuco-form of the dye and growth of dye background values [23], was avoided;
(3) staining of the permeabilized spores with malachite green; at this step; PSDVB particles (20–50 μg/ml sample) were

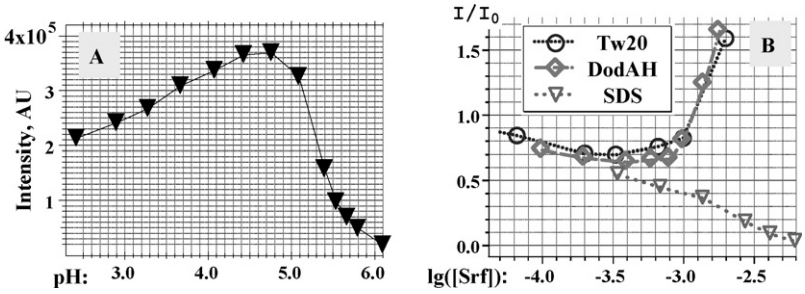


Fig. 4. (A) Effect of pH on the malachite green (MG)–PMAA fluorescence enhancement. *Conditions*: [MG] = 200 nM, [PMAA] = 20 mM_R in 20 mM citrate; pH adjusted with 3 M NaOH. Emission intensities 627:646 nm are plotted vs. pH values. The latter were determined in the samples of the same content yet containing no dye. (B) Effect of example surfactants Tween 20 (Tw20, neutral), dodecylamine × HCl (DodAH, cationic) and sodium lauryl sulfate (SDS, anionic) on 626:646 nm emission of MG–PMAA. *Conditions*: [MG] = 400 nM, [PMAA] = 20 mM, in 20 mM citrate, pH 4.0; [Srf] is molar concentration of a surfactant. Relative intensities I/I_0, where I_0 is the emission intensities of the corresponding reference samples with no surfactants added, are plotted vs. molar concentrations of surfactants.

added for more efficient spore trapping in the samples; steps 1–3 were performed *in situ*;

(4) washing off the non-specifically bound dye, with SDS-containing buffer at pH not exceeding ~6 (separation of the stained spores from washing solution can be performed by centrifugation or microfiltration);

(5) extracting/recovery the spore-bound dye under acidic solution (e.g., citric acid containing EDTA).

The protocol requires only standard laboratory equipment and can be performed over ~1–2 h. The protocol ensured almost quantitative uptake of malachite green by permeabilized spores, consistently with earlier reported staining responses for TAM dyes [14]. In case of UV–vis detection of the spore-recovered malachite green, the lowest detection limit of the method attained here was ~2×10^6 spores per sample (~1 ml), i.e., is comparable with that of DPA method reported earlier. At the same time, the reported MG uptake assay shows much higher tolerance toward inorganic anions and surfactants (i.e., potential assay masking factors) than the existing methods for spore detection [1]. The developed assay protocol demonstrates high robustness and consistency related to the tested endospore model, *B. subtilis var.* "*niger*" (Fig. 5).

3.5. Use of malachite green–PMAA fluorescence enhancement for detection of bacterial endospores in aqueous samples

Addition of PMAA to the extraction solution (20 mM citric acid), in the reported malachite green assay for spore detection, enhanced the assay sensitivity up to ~10^4 spores per sample (Fig. 2), i.e. 100 times more, compared to that of the dipicolinate assay. As low as 1 mM (per residue) of PMAA in the solution enables efficient emission enhancement of malachite green. Practically, higher concentrations of PMAA (5–10 mM per residue) were used. Before measurements, the pH of the solution was raised up to 4.5–5 with sodium citrate, in order to enhance the emission signal, in accordance with the determined MG–PMAA emission pH optimum (Fig. 4A). For the highest signal-to-background ratio, the excitation wavelength 430 nm was preferred. The relatively large (220 nm) Stocks distance associated with the $S_2 \rightarrow S_0$ transition of MG makes this dye especially suitable for fluorimetric analysis of light scattering samples, such as spore suspensions. The presence of surfactants (<0.05%), EDTA and inorganic anions in the original sample did not interfere with the assay. The assay also tolerates the presence of polystyrene and silica microparticles in the samples (10:1–100:1 excess by dry weight, as related to the dry weight of the suspended spores; not shown). Thereby the described procedure might be suitable for the detection of spores in the presence of environmental particles (e.g., dust).

Earlier crystal violet (CV) was reported to display demonstrate high affinity for permeabilized spores [14] and to undergo sharp emission enhancement in PMAA hypercoiled environment at pH < 5 [17]. We also found that another cationic stain employed earlier in dye elution assays for cell attachment, neutral red [24], demonstrate similar emission enhancement in PMAA hypercoil (not shown). Therefore, crystal violet, and neutral red were successively tested here as uptake probes for spore quantitation according to Scheme 1, with the correspondingly adjusted optical parameters of fluorometric assay (not shown).

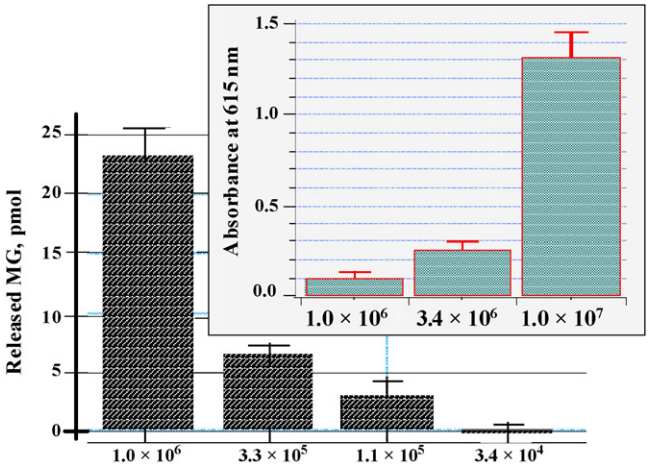


Fig. 5. Fluorescent PMAA–MG elution assay with *B. subtilis* spores—a typical plate reader experiment. The amounts of malachite green eluted from spores were calibrated against standard dye dilutions. Endpoint emission values at 428:655 nm (cutoff 630 nm) vs. dye amounts) were corrected for background control series (the corresponding spore-free PSDVB suspensions). Inset: a typical UV–vis malachite green assay with PMAA-free citrate-EDTA elution buffer. All samples are proceeded in triplicates, SEM bars are shown with the plots.

However in the specific case of suspended bacterial endospores, malachite green oxalate proved to be the most suitable reagent. This dye displayed highest staining response, best specificity toward spores and lowest staining and optical background in the assay conditions compared to the other cationic stains tested.

4. Discussion

Malachite green is known as an efficient endospore stain that undergoes quantitative uptake by heat-shocked, acid-permeabilized spores. Previously, spectrophotometric (UV–vis) quantitations of dye uptake by cells has been used only for crude estimation of cell count in surface-attached cultures. In the current work, the high capacity of acid-pretreated heat-shocked bacterial spores for specific uptake of malachite green [14] was employed for their detection in aqueous suspension, in the presence of masking agents (anions, surfactants, polystyrene and silica microparticles). Among attractive features of this approach is the possibility of separate detection of dormant endospores in the presence of other microbial forms (predominantly lysed during the "hot staining" treatment).

Spectrophotometric detection of spore-bound malachite green stain enables crude quantitation of suspended bacterial spores (Fig. 5, inset). Nevertheless, the UV–vis determination of spore-released malachite green did not provide count sensitivity below 10^6 spores/ml. Thereby the final design of our spore detection assay employs PMAA-enhanced fluorescence of malachite green specifically taken up by spores and then *in situ* re-partitioned, after washing off the background dye, into the PMAA microphase. The demonstrated high affinity of interactions between MG and hypercoiled PMAA form enables efficient repartitioning of this dye, to the PMAA hypercoil from the binding sites within bacterial spores, under the reported assay conditions. Thereby, PMAA can be used for quantitative *in situ* extraction of the stain specifically absorbed by spores.

Compared to dipicolinate assay for spore detection [7], the MG–PMAA assay is ∼100 times more sensitive (∼10^4 spores/ml limit of detection), employs a relatively simple protocol and tolerates various soluble and particulate impurities expected to mask spores in environment-collected samples, such as buffers, traces of surfactants, proteins and non-relevant particulates. These characteristics of the assay make it feasible for laboratory and field applications in a number of venues that may demand fast detection of spore-forming bacterial species in the environment. In particular, the use of the PMAA additive as emission enhancer for malachite green and other cationic stains might generally improve the sensitivity of existing colorimetric bioassays based on dye elution; e.g., in determination of cell adhesion or cytotoxicity.

Acknowledgment

Financial support for this work and the cultures of *B. subtilis* spores spore were provided by the U.S. Army Research Laboratory.

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