Plate reader spectroscopy as an alternative to atomic absorption spectroscopy for the assessment of nanoparticle cellular uptake

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ABSTRACT

Fundamental studies investigating the biological effects induced by nanoparticles (NPs) explicitly require the correct assessment of their intracellular concentration. Ultrasensitive atomic absorption spectroscopy (AAS) is perceived as one of the gold standard methods for quantifying internalized NPs. Besides its limitation to metal-based NPs though, AAS also requires specific infrastructure and tedious sample preparation and handling, making it time-consuming and cost-intensive. Herein we report on a reliable, rapid, and affordable alternative to AAS – plate reader spectroscopy (PRS), which offers an accessible option for everyday laboratory use without sophisticated instrumentation. Our results demonstrate, that following a proper methodological approach, data on intracellular concentration of NPs obtained by PRS are fully comparable to AAS results. Specifically, the intracellular concentration of magnetite NPs coated with sodium oleate and bovine serum albumin in human alveolar A549 cells was assessed by PRS and AAS in parallel, with a remarkable correlation coefficient of $R = 0.9914$.

1. Introduction

The growing interest in the use of nanoparticles (NPs) for medical purposes is constantly increasing the demand for simple methods enabling their reliable intracellular quantification. Knowledge of the intracellular concentration of NPs is especially important as most of the biological effects manifest in a dose-dependent manner [1, 2, 3, 4, 5, 6]. Since there is no commonly accepted approach for studying NPs in a biological environment, it is hardly possible to derive generalizable conclusions from different studies. A plethora of analytical methods is employed to measure the cellular uptake of NPs. While microscopy methods dominate in qualitative evaluation, spectroscopy methods including atomic absorption spectroscopy (AAS) [7, 8], inductively coupled plasma-mass spectrometry [9, 10, 11], inductively coupled plasma-atomic emission spectroscopy [12], flow cytometry or confocal imaging [2, 13, 14, 15] are preferred for quantitative assessment.

AAS is a destructive method commonly used for qualitative and quantitative analysis of metallic elements in the samples [16, 17, 18]. It enables ultrasensitive detection of elements across a wide range of concentrations with excellent repeatability, requires as short as 10–15 s for a single measurement, and offers a possibility for automatization [19]. On the other hand, AAS requires expensive instrumentation with skilled operators, and its use is often limited due to the time-consuming sample preparation as well as the requirement for relatively large volumes of samples (0.5–5.0 mL), which makes it impractical, especially for screening studies [2, 16].

Regardless of all the indisputable benefits offered by AAS, these drawbacks open up space for alternative analytical methods with adequate sensitivity that are more suitable for daily laboratory practice.

One of the rapid and affordable methods enabling the quantification of substances is plate reader spectroscopy (PRS), suitable for absorbance measurements in the UV-VIS spectrum. Plate readers are accessible in most laboratories and are widely used for screening experiments as they allow for measuring multiple samples at once [20].

Herein we investigated the suitability of PRS for routine quantification of intracellular concentration of NPs based on their absorbance, as an accessible alternative to AAS. Within this study, bovine serum albumin (BSA) and sodium oleate (SO) coated magnetite NPs (BSA–SO–MNPs)
were used, as they are biocompatible and have favorable toxicity profiles [21, 22, 23, 24]. The metallic core enabled parallel quantification of NPs by AAS. Since lungs are well-known to accumulate iron oxide NPs [25, 26], intracellular uptake of BSA–SO–MNPs was evaluated in human alveolar epithelial cancer cell line A549.

2. Materials and methods

2.1. Cell culture

A549 (ATCC® CCL-185™) cells were obtained from American Type Culture Collection (USA). A549 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) in low glucose (1 g/L) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin (all from Gibco, Thermo Fisher Scientific, USA). Cells were cultured in a humidified atmosphere with 5% CO2 at 37 °C.

2.2. Magnetite nanoparticles

In this study, spherical NPs with Fe3O4 core coated with SO and BSA (Table S1) were used. BSA–SO–MNPs were prepared, characterized, and kindly provided by Vlasta Zavisova, Ph.D., Institute of Experimental Physics, SAS, Kosice, Slovakia [27].

2.3. Exposure of cells to BSA–SO–MNPs

For PRS measurements, cells were seeded onto 6-well plates (Ø = 35 mm) at a density of 2 × 103 cells/well. After 24 h, cells were pre-treated in a culture medium with 2% FCS for 24 h. Cells were then exposed to various non-toxic concentrations [25, 26] of BSA–SO–MNPs (herein expressed as µg of Fe3O4 per mL) ranging between 0-116 µg Fe3O4/mL for 5 h in culture medium with 2% FCS. After exposure, cells were washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific, USA), trypsinnzed, counted, and pelleted (200 × g, 5 min). The supernatant was removed, and the pellet was lysed in 120 µL lysis buffer containing RIPA (Serva, Germany) with SDS (Merck, USA) added to a final concentration of 1%. Cell lysates were homogenized by brief sonication using QSonica Q55 Sonicator (Qsonica, USA).

For AAS analysis, cells were seeded on Petri dishes (Ø = 100 mm) at the density of 1 × 103 cells/dish. After 24 h, cells were pre-treated in the medium with 2% FCS for 24 h. Cells were then exposed to various non-toxic concentrations [25, 26] of BSA–SO–MNPs (herein expressed as µg of Fe3O4 per mL) for 5 h in the culture medium with 2% FCS. After exposure, cells were washed with PBS, trypsinnzed, counted, pelleted (200 × g, 5 min), and digested using 500 µL of 65% HNO3 (Centralchem, Slovakia) in an ultrasonic bath at 85 °C for 2 h. The digests were then diluted in 2% HNO3 solution.

2.4. UV-VIS spectroscopy

The representative UV-VIS absorption spectrum of BSA–SO–MNPs was assessed using the Shimadzu UV-1650 PC UV-VIS spectrometer (Shimadzu Corporation, Japan) for the concentration of 35.5 µg Fe3O4 per mL.

The absorption maximum of BSA–SO–MNPs was 273 nm. To avoid cross-interference with the intracellular protein fraction (~280 nm), a wavelength of 350 nm was arbitrarily chosen to determine the calibration curve. In case of transmittance of a sample was below 10%, it was diluted and re-measured.

2.5. Plate reader spectroscopy

Based on the UV-VIS results, the absorbance of samples was recorded at 350 nm and calibration curves for BSA–SO–MNPs were determined in different relevant solutions using xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, USA). The limit of detection (LOD) and limit of quantification (LOQ) of PRS method derived from the calibration curve were 5.113 mg/L and 15.338 mg/L, respectively. For PRS analysis we used a 96-well plate with a flat bottom with 100 µL of the sample per well. Each sample was analyzed in triplicate. The assessed absorbance was used to calculate the concentration of BSA–SO–MNPs based on the calibration curve. During the measurements, signals were corrected to the negative control represented by the cell lysate of non-treated cells. Finally, the intracellular concentration of NPs was normalized to the volume of the sample and number of cells and expressed as pg Fe3O4 per cell.

The cellular uptake of BSA–SO–MNPs in the samples was calculated according to the following formulas:

\[ \text{pg Fe}_3\text{O}_4 \text{ per cell} = \frac{\text{absorbance of the sample in well at 350 nm}}{a} \times b \]

where \( a \) and \( b \) are constants from calibration curve: \( y = ax + b \)

2.6. Atomic absorption spectroscopy

The analysis was carried out using Varian AA240FS (Varian Inc., USA) atomic absorption spectrometer, equipped with a flame burner. AAS was adapted to quantify the amount of Fe in the samples with the following setting: wavelength 248.3 nm, slit width 0.2 nm, flame type: acetylene-air, flow: 2.0 L/min for acetylene, and 13.5 L/min for air, deuterium background correction, method of the calibration curve in the range 0.1–10 mg/L. The limit of detection (LOD) and limit of quantification (LOQ) for the AAS instrument were 0.002 mg/L and 0.005 mg/L, respectively. LOD and LOQ for the AAS method were 0.007 mg/L and 0.025 mg/L, respectively. All samples were analyzed in triplicate.

The amount of Fe was corrected to the signal from un-exposed control cells and used to calculate the amount of magnetite expressed as pg Fe3O4 per cell.

3. Results

3.1. Assessment of the absorption spectrum of BSA–SO–MNPs by UV-VIS spectroscopy

To quantify NPs in samples using PRS, it was first necessary to determine the absorption spectrum of BSA–SO–MNPs and to determine the appropriate wavelength for a reliable assessment of their absorbance. Figure 1 shows the characteristic absorption spectrum of BSA–SO–MNPs in water in the wavelength range 265–765 nm with an absorption maximum at 273 nm.

Concerning the absorption maximum of nucleic acids and proteins between 260-280 nm, the wavelength of 350 nm was arbitrarily selected for quantitative analyses, as it falls outside the cross-interference region, while still providing meaningfully high absorbance (64% of the absorption maximum) for reliable measurement of BSA–SO–MNPs concentration in the samples.

The suitability of wavelength 350 nm to determine of BSA–SO–MNPs concentration in samples was evaluated through the linearity of absorbance versus NPs concentration in terms of the Beer-Lambert law. Since linear regression of the obtained scatter had a coefficient of determination \( R^2 = 0.9998 \) (Figure 1, right), wavelength 350 nm proved to be suitable for measuring the absorbance of NPs and calculating their concentration. Based on this finding, the intracellular concentration of BSA–SO–MNPs was calculated based on the absorbance measured by PRS at 350 nm. Determination of calibration curves for BSA–SO–MNPs in different solutions by PRS was also determined (Figure S1).

3.2. Comparison of PRS and AAS in the quantification of intracellular concentration of BSA–SO–MNPs

To assess PRS as a suitable alternative method to AAS, data on the intracellular concentration of BSA–SO–MNPs in exposed A549 cells
obtained by PRS and AAS were compared head-to-head. Figure 2 shows the intracellular concentration of BSA–SO–MNPs in A549 cells determined by PRS and AAS for different applied NPs concentrations. Data showed an excellent correlation of results from both methods (Figure 3) with a coefficient of correlation $R = 0.9914$. Moreover, the overlap of theoretical and experimental linear regressions indicates, that the absolute values obtained by PRS fully correspond with those obtained by the reference AAS method.

Figure 1. Characteristic UV-VIS spectrum of BSA–SO–MNPs. Calibration curve of BSA–SO–MNPs in water assessed by UV-VIS spectroscopy at 350 nm (inset).

Figure 2. Intracellular concentration of Fe$_3$O$_4$ assessed by AAS and PRS for various applied concentrations of NPs: 0, 23, 46, 69, 93, 116 μg Fe$_3$O$_4$/mL. Data are given as mean values ± SEM from independent experiments ($N = 3$).

Figure 3. Correlation of results on intracellular concentration of Fe$_3$O$_4$ assessed by PRS and AAS — gray diagonal represents the ideal correlation of data; dashed line represents the correlation of experimental data ($R = 0.9914$). The 95% confidence interval is shown in red. Data are given as mean values ± SEM from independent experiments ($N = 3$). The correlation was calculated using the GraphPad Prism software (GraphPad Software, USA) with the Spearman correlation method, with $p$-value = 0.0028.
4. Discussion

Our study revealed an excellent correlation between data from AAS and PRS regarding the assessment of the intracellular concentration of NPs. Unlike AAS which measures the absorbance of Fe, PRS measures the absorbance of the entire nanoparticle, including its coating. As long as the PRS measurement results are corrected for untreated cells, the results are not biased by endogenous Fe. In addition, compared to AAS, PRS is more flexible in terms of the measured analyte, as it does not require a metal core and possibly enables the quantification of NPs based on the absorbance of the coating alone. In the latter case, it is necessary to ensure that the coating used is stable in the biological environment. For daily routine practice, as far as the analyzed NPs absorb light in the UV-VIS spectrum and it is possible to select a wavelength that does not interfere with other components in the measured samples, PRS could be used as fast cost-effective full-fledged alternative to AAS for determination of intracellular uptake of NPs.

Declarations

Author contribution statement

Barbora Svitkova, Michal Selc, Veronika Nemethova, Filip Razga: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alena Gabelova: Contributed reagents, materials, analysis tools or data.

Monika Ursinyova: Performed the experiments.

Andrea Babelova: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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