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ORIGINAL PAPER

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Quantitative detection of asbestos fiber in gravelly sand using elastic body-exposure method

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Abstract Chrysotile or crocidolite colloidal solution containing donor plasmid DNA and *Escherichia coli* cells was subjected to elastic body friction. These acicular clay minerals mediated *E. coli* antibiotic resistance plasmid transformation. Other clay minerals had no effect on *E. coli* transformation. The number of *E. coli* transformants was counted after elastic body exposure with various crocidolite concentrations. There was a correlation between the number of *E. coli* transformants and crocidolite concentration (between 40 and 1,000 ng/ml). A mixture consisting of sea sand and crocidolite was utilized as a model for quantitative detection of asbestos in gravelly sand. With sea sand containing 0.15–15 mg of crocidolite, a correlation between crocidolite concentration and the number of colonies derived from *E. coli* transformants was observed. This indicates that measurement of asbestos is possible even when the asbestos sample includes gravelly sand. Fluorescence microscopic observation of crocidolite colloidal solution indicated that crocidolite was present as spherical aggregates having diameters of 6–9 μm. Thus, the number of transformants correlated with that of 6–9 μm crocidolite aggregates.

Keywords Crocidolite · Chrysotile · Friction · Plasmid · Elastic body exposure

Introduction

Asbestos is the general commercial term for a group of naturally occurring hydrated silicates that crystallize in a fibrous manner; asbestos minerals are classified as serpentine and amphibole fibers [10]. One of the most common serpentine fibers, chrysotile [$Mg_6Si_4O_{10}(OH)_8$], accounts for over 90% of the world's production of asbestos. Amphibole asbestos fibers are more chemically diverse and less important for industry; they include crocidolite [$Na_2(Fe^{3+})_2(Fe^{2+})_3Si_8O_{22}(OH)_2$] and amosite [$(Fe,Mg)_7Si_8O_{22}(OH)_2$] asbestos. Asbestos is attractive for industrial use due to its resistance to heat and chemicals, high tensile strength and low cost when compared with similar man-made materials. However, a large body of epidemiological and experimental evidence clearly indicates that asbestos fibers are carcinogenic [1, 5, 8, 19]. Although the use of spray-on asbestos as a fireproofing material or insulation has been banned in the USA as well as in several European countries, asbestos is currently incorporated in cement construction materials, friction materials, jointing and gaskets, asphalt coats and sealants [20].

Nonetheless, there is currently no cogent mechanism relating asbestos-induced oncogenesis to changes in DNA at the molecular level. Several hypotheses have been proposed to explain how asbestos causes mutation in animal cells, but few have been studied extensively [16]. Recent studies on asbestos in cell-free systems have demonstrated by electron spin resonance that chrysotile, crocidolite and amosite asbestos generate active oxygen species in the presence of H_2O_2 or physiological saline [21]. Asbestos complexes have significant levels of ferric iron on their surfaces. Under these circumstances, Fe^{2+} on the surface of asbestos appears to drive a modified Haber-Weiss (Fenton) reaction that results in the production of the toxic hydroxyl radical from H_2O_2 and O_2^-. Active oxygen species can react with DNA, causing DNA strand breaks [18] and base modifications [6] as well as the induction of cellular oxidative stress. Asbestos may have multiple effects in mutation, but the molecular mechanisms of asbestos-induced mutagenesis remain unclear. Asbestos fibers were previously used for industrial materials in many fields. However, when asbestos fibers were found to be a carcinogen through epidemiological research, their uses were restricted. Accurate methods for determining environmental

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asbestos levels must therefore be established, as asbestos is a dangerous pollutant.

The current measurement method is the phase-contrast microscopy method prescribed in method no. 7400 of the National Institute for Occupational Safety and Health [14, 15, 17]. This method, which involves manually counting fibers in specimens, is time consuming and requires substantial expertise. Furthermore, the values obtained using this method are subject to large errors. For example, if the counting time is different, values obtained in the same specimens by the same technician differ. Lack of reliability and objectivity in these counted values has been reported [9]. Transmission electron microscopy of air samples is also essential for identification and quantification of finer asbestos fibers [4]. However, the detection of finer asbestos by microscopy is impossible when the sample includes gravelly sand, which consists of several clay mineral species. These measurement methods were developed for researchers; however, many counting facilities cannot readily apply them, as they are expensive.

In a previous study, we examined whether chrysotile asbestos particles are able to mediate the transformation of *Escherichia coli* cells by exogenous plasmid DNA [24]. A suspension of recipient *E. coli* cells in the stationary phase, along with chrysotile asbestos fibers and pUC18 donor DNA, was spread over the surface of an LB agar plate (elastic body) using a stir stick, after which the intracellular uptake of plasmid DNA by *E. coli* cells was observed. Optimal conditions resulted in 10^6 transformants per microgram of pUC18 DNA. A drastic physical change due to 'quick drying of agar plate surface', during which time the cell–chrysotile suspension was exposed to agar, was required for transformation to occur (elastic body-exposure method). Electron microscopy of the agar-exposed cell–chrysotile suspension revealed penetration of the *E. coli* cell membrane by chrysotile fibers. It has been suggested that transformation of *E. coli* with plasmid DNA might be the result of penetration by chrysotile fibers onto which plasmid DNA is adsorbed [25]. The present study examines whether the elastic body-exposure method can be applied to the determination of asbestos concentration in gravelly sand with the aim of replacing the conventional method for asbestos determination.

Materials and methods

Chemicals

Chrysotile and crocidolite were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and Kanto Chemical Co., respectively. Bentonite, talc and magnesium oxide were obtained from Wako. Kaolinite was from Nacalai Tesque, Inc. (Kyoto, Japan). Glass wool and rock wool were obtained from architectural waste. Ampicillin and agar were from Wako and Nacalai, respectively.

Bacterial strain and plasmid

Escherichia coli JM109 (el4$^-$, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, Δ(*lac-pro*AB), [F′, *tra*D36 *pro*AB, *lac*I^q ZΔM15]) [22] was used as the recipient cell line. pUC18 [22] was used as exogenous donor DNA to determine the efficiency of clay mineral-mediated transformation.

Preparation of aqueous colloidal solutions of clay minerals

Clay minerals (chrysotile, crocidolite, bentonite, kaolinite, glass wool, rock wool, talc) and magnesium oxide were isolated using a sedimentation procedure [11]. A 0.4 mg sample of each clay mineral suspended in 40 ml of deionized distilled water was vigorously shaken for 10 min (1,500 rpm EYLA cute mixer CM-1000) and then centrifuged at 2,000 × *g* for 2 min. After centrifugation, the upper phase was discarded in order to remove any large particles. The clay mineral pellet was then re-suspended in 40 ml of deionized distilled water and vigorously shaken for 10 min (1,500 rpm). The upper phase obtained by centrifugation at 2,000 × *g* for 2 min was collected, autoclaved at 121 °C for 15 min and used as colloidal solution containing clay mineral dispersed in deionized distilled water. The concentration of clay particles within the aqueous clay mineral colloidal solution was estimated to be 40–50 μg/ml.

Preparation of cell–clay mineral suspension

Escherichia coli JM09 were cultured in Luria-Bertani (LB) broth [13] for 18 h at 37 °C with aeration (150 rpm). Cells were used when cell density reached an OD550 of 3.0, as determined by spectrophotometry. A 500 μl (∼ 3.0×10^8 cells) aliquot of the *E. coli* culture was centrifuged for 2 min at 9,000 × *g* at room temperature. The *E. coli* cell pellet was then re-suspended in 475 μl of aqueous clay mineral colloidal solution plus 0.5 ng of pUC18 followed by 25 μl of 4 M NaCl (cell–clay mineral suspension).

Standard protocol for elastic body-exposure method

The surfaces of 2% agar LB plates (8.5 cm in diameter) were rapidly dried in a clean room to remove all visible condensation before spreading the cell–clay mineral suspension. A 50 μl aliquot of each suspension was spread on each LB agar plate. Without disrupting the agar, pressure was applied to the surface of the plate for 60 s using a plastic stir stick (SARSTEDT, Germany) on an automated turntable, T-Au (Iuchi Co., Osaka, Japan), set at 90 rpm. We defined this treatment as elastic body exposure. The vertical reaction force of the

stir stick was maintained at 40 g by the apparatus [23], which was designed for elastic body exposure. The various cell–clay mineral suspensions exposed to LB agar plates supplemented with 50 μg/ml of ampicillin were used. After incubation for 18–20 h at 37 °C, the number of colonies demonstrating antibiotic resistance (indicating transformation) was counted. Transformation efficiency was expressed as the number of transformants observed per microgram of plasmid DNA.

Measurement of asbestos concentration in sea sand

Sea sand was washed with distilled water and fine sieved with 30 mesh (aperture 500 μm) screen. Sieved fine sea sand was sterilized at 60 °C for 18 h. Crocidolite was dissolved in distilled water at a final concentration of 5 mg/ml and then autoclaved at 121 °C for 15 min. One gram of sterilized fine sea sand was suspended in 3 ml of crocidolite solution with a concentration of 0.05–5 mg/ml.

This suspension was then vigorously shaken for 10 min (1,500 rpm, EYLA cute mixer CM-1000) and centrifuged at $2{,}000 \times g$ for 2 min. The upper phase obtained by centrifugation at $2{,}000 \times g$ for 2 min was collected, filtered through a 30 μm filter (Millipore nylon net filters NY30) and used as colloidal solution containing asbestos dispersed in deionized distilled water. *E. coli* JM09 was cultured in Luria-Bertani (LB) broth [13] for 18 h at 37 °C with aeration (150 rpm). Cells were used when cell density reached an OD550 of 3.0, as determined by spectrophotometry. A 500 μl ($\sim 3.0 \times 10^8$ cells) aliquot of the *E. coli* culture was centrifuged for 2 min at $9{,}000 \times g$ at room temperature. The *E. coli* cell pellet was then re-suspended in 475 μl of colloidal solution containing asbestos plus 20 ng of pUC18 followed by 25 μl of 4 M NaCl (cell–clay suspension). Cell–clay mineral suspensions were exposed to LB agar plates supplemented with 50 μg/ml of ampicillin. After incubation for 18–20 h at 37 °C, the number of colonies demonstrating antibiotic resistance (indicating transformation) was counted.

Fluorescence microscopy

The aqueous crocidolite colloidal solution (50 μg/ml) was mixed with 0.5 μM synthetic DNA oligomers (5′-CTA CCG CTT CGT GGA GCA GCC CGC CC-3′) tagged at the 5′-end with fluorescein isothiocyanate (FITC), followed by direct observation using fluorescence microscopy. Microscopic analysis was performed using a fluorescence microscope (ZEISS Axioskop 2plus, Germany). For fluorescence studies, filter blocks (exciter 460–500 nm; dichroic mirror 505 nm; emitter 510–560 nm) were used to visualize FITC (ZEISS). Experiments were documented using a Zeiss digital camera (AxioCam MRc5).

Scanning electron microscopy

Crocidolite particles within the crocidolite colloidal solution were collected by centrifugation at $10{,}000 \times g$ for 30 min, after which they were dried at 80 °C for 24 h. After being platinum palladium sputtered (140–160 Å), the crocidolite fibers were examined using a scanning electron microscope (Hitachi S-800M) at an accelerating voltage of 20 kV.

Results and discussion

Which clay minerals enable to transport plasmid DNA to *E. coli* cells?

Environmental gravel comprises various clay minerals. Thus, when determining asbestos concentration in gravel, the effects of other clay minerals on *E. coli* cells, plasmids and elastic body exposure must be considered. *E. coli* cells suspended in aqueous asbestos (chrysotile, crocidolite) colloidal solution containing plasmid DNA (pUC18) were subjected to elastic body exposure, and transformation of *E. coli* by incorporation of plasmid DNA was confirmed. We then examined whether clay minerals, with the exception of asbestos, are able to introduce plasmid DNA into *E. coli* cells.

Kaolinite and bentonite are the most common soil clay minerals in many parts of the world. Clay minerals in the kaolinite group are characterized by a 1:1 tetrahedral:octahedral sheet. Chrysotile is similar to kaolinite in that they are both 1:1 phyllosilicates. Positive ions (e.g., Al^{3+}, Mg^{2+}) are present in the center of the octahedron of kaolinite and chrysotile, respectively. Kaolinite did not mediate the transformation of *E. coli*, despite its high similarity with chrysotile. The prominent difference between the two is crystal formation. Kaolinite and chrysotile form thin hexagon plate crystals and acicular crystals, respectively [2, 3]. Bentonite has a high content of sodium montmorillonite and smaller portions of quartz, feldspar, etc. Neither bentonite nor kaolinite enables plasmid transport into *E. coli* cells. Talc is a type of high water content magnesium silicate that is formed via connections between oxygen and hydroxyl groups. The structural formula of talc [$Mg_3Si_4O_{10}(OH)_2$] more closely resembles that of chrysotile than kaolinite. The crystal form of the talc powder is tabular, although acicular talc has also been identified. However, talc had no effect on *E. coli* transformation.

The physical properties of glass wool and rock wool, which are produced artificially as an asbestos substitute, resemble those of chrysotile and crocidolite, but there are differences in fiber thickness. The diameter (1–2 μm) of glass wool and rock wool is larger than that of chrysotile. The effects of gene introduction into *E. coli* could not be examined with glass wool and rock wool. Chrysotile fiber contains numerous MgO molecules, but

the effects of gene introduction into *E. coli* could not be examined with pure MgO (white crystal particles).

These results show that only asbestos species, such as chrysotile and crocidolite, are able to mediate *E. coli* transformation by plasmid DNA (Table 1). Crystal form and size are more important than the structural formula of clay minerals for gene introduction. Acicular clay minerals having suitable diameter and hardness are essential for *E. coli* transformation through elastic body exposure. Asbestos species induce *E. coli* transformation via the application of sliding friction. These results indicate that even if asbestos is intermingled with glass wool, rock wool or other clay minerals, such as kaolinite, the amount of asbestos in the gravel would correlate to the number of *E. coli* transformants obtained by the elastic body-exposure method.

Minimal crocidolite levels in colloidal solution required for achieving gene introduction

In order to achieve measurement of asbestos concentration in gravel using elastic body exposure, the detection limit under laboratory conditions must be determined. The crocidolite reagent is marketed as pulverulent, and its handling for quantification is simple. The number of *E. coli* transformants through the elastic body exposure at various crocidolite concentrations was counted. As shown in Fig. 1, there is a correlation between the number of *E. coli* transformants and crocidolite concentration (between 40 and 1,000 ng/ml). When plotted, the number of *E. coli* colonies exhibiting antibiotic resistance and the concentration of crocidolite gave a regression line expressed as $y = 0.585x + 49.33$, where the minimum concentration of crocidolite was 40 ng/ml. Increased amounts of plasmid DNA were sensitized to crocidolite concentration, even at less than 40 ng/ml.

Possible quantification of asbestos in sea sand

The concrete from which buildings are constructed comprises cement, sand derived from sea or river bed and large gravel. Therefore, concrete waste materials should contain large quantities of sea or river sand. Mixing asbestos with sand was thus considered to be an ideal model for measurement of asbestos in gravelly sand. By preparing a mineral colloidal solution and counting the number of transformants following elastic body exposure, we examined the possibility of the environmental asbestos fiber determination. As shown in Fig. 2, when 1 g of sea sand contained 0.15–15 mg of crocidolite, there is a correlation in the quantity of crocidolite and the number of *E. coli* transformants following elastic body exposure. Thus, estimation of asbestos concentration in sea sand is possible. Crocidolite and chrysotile gave the same efficiency of *E. coli* transformation. Owing to the acicular form of asbestos, it acts as a vehicle for plasmids during elastic body exposure. Measurement of asbestos concentration in environmental gravelly sand appears to be possible through the above-mentioned procedure. As shown in Fig. 2, correlation between crocidolite concentration and the number of transformants provided the following formula,

$$y = 33.75x + 14.34, \quad (1)$$

where y is the number of transformants (cfu/plate) and x the crocidolite concentration (mg/g). If gravelly sand is treated by the above procedure and gives150 colonies on a single plate, asbestos concentration in gravelly sand would be 4.0 mg/g. However, this estimation method is not suitable for asbestos levels of less than 100 μg/g. The proposed protocol for measurement of asbestos concentration in environmental gravelly sand using the elastic body-exposure method (*E. coli* transformation method) is shown in Figs. 3 and 4. First, *E. coli* cells and plasmids are dissolved in an aqueous clay mineral colloidal solution prepared from a sand–crocidolite mixture (known crocidolite concentration), followed by elastic body exposure. A standard curve is prepared by correlation between crocidolite concentration and the number of *E. coli* transformants (Fig. 3). Next, *E. coli* cells and plasmids are dissolved in clay mineral colloidal solutions prepared from gravelly sand, followed by elastic body friction, and the number of *E. coli* transformants is counted. Finally, asbestos concentration in environmental gravelly sand is determined based on the standard curve (Fig. 4).

Table 1 Clay mineral's ability to bring exogenous plasmid DNA to *E. coli* cells

Clay minerals	Structural formula	Transformant (cfu)/plate
Bentonite	$(Na,Ca)_{1/3}(Al, Mg)_2[(OH)_2Si_4O_{10}]\cdot nH_2O$	ND
Kaolinite	$Al_2Si_2O_5(OH)_4$	ND
Chrysotile	$Mg_6Si_4O_{10}(OH)_8$	200 ± 12
Crocidolite	$Na(Fe^{2+} > Mg)_3Fe_2^{3+}Si_8O_{22}(OH)_2$	210 ± 10
Glass wool	SiO_2 artifact	ND
Rock wool	Artifact	ND
Talc	$Mg_3Si_4O_{10}(OH)_2$	ND
Manganese oxide	MgO	ND

ND not detected

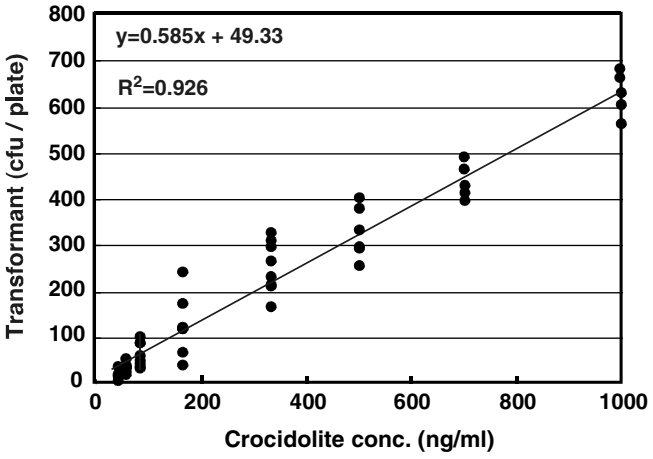

Fig. 1 Determination of crocidolite in aqueous colloidal solution by elastic body-exposure method and correlation between crocidolite concentration and the number of *E. coli* transformants

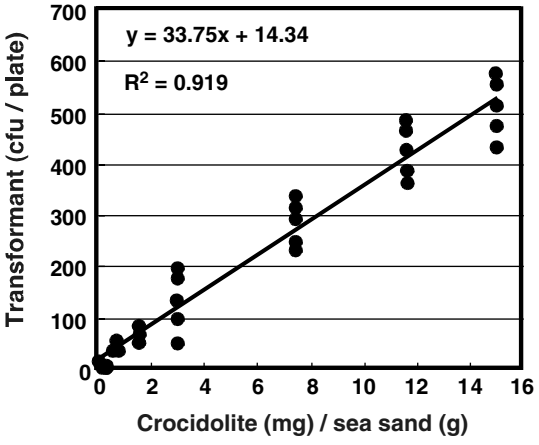

Fig. 2 Correlation between crocidolite concentration in gravelly sand and number of *E. coli* transformants. Clay mineral solution prepared from gravelly sand containing crocidolite asbestos was mixed with *E. coli* cells and subjected to elastic body exposure

Morphological observation by microscopy

The crocidolite fibers in aqueous crocidolite colloidal solution ranged from 0.05 to 0.12 μm in diameter and from 0.5 to 3.0 μm in length, as determined using scanning electron microscopy (SEM) (Fig. 5). Crocidolite and chrysotile (data now shown) particles were acicular. Physical properties, such as aggregation of clay particles, are different in the wet and dry states. Hence, the physical state of particles and the dispersion of crocidolite particles in the colloidal solution must be ascertained. Discrimination of acicular crocidolite by light microscopy is extremely difficult, as the refractive index of crocidolite is similar to that of water [7, 12]. To observe the behavior of crocidolite particles, we used fluorescence microscopy to identify crocidolite-labeled FITC-conjugated DNA. As shown in Fig. 5, fluorescence microscopy was used to examine the emission of green fluorescence by individual crocidolite particles. It was not possible to identify individual crocidolite particles, only large chrysotile aggregates (5–10 μm). A previous report found that aggregates (6–9 μm) of acicular particles were involved in *E. coli* transformation [23]. Accordingly, there is an apparent correlation between the number of transformants and acicular particle aggregates.

In many cases, asbestos intermingles with other clay minerals, and thus detection of asbestos by electron microscopy is very difficult. X-ray diffraction quantitative analysis of fibrous substances like asbestos is strongly influenced by systematic errors due to the presence of other clay minerals. X-ray diffraction is thus useful to determine the type of asbestos fiber present, but is unsuitable for quantitative analysis. Quantitative determination of asbestos using the elastic body-exposure method is possible, despite the presence of large quantities of clay minerals. Consequently, this novel method, which is capable of asbestos quantification in both gravelly sand and dust, overcomes the deficiencies

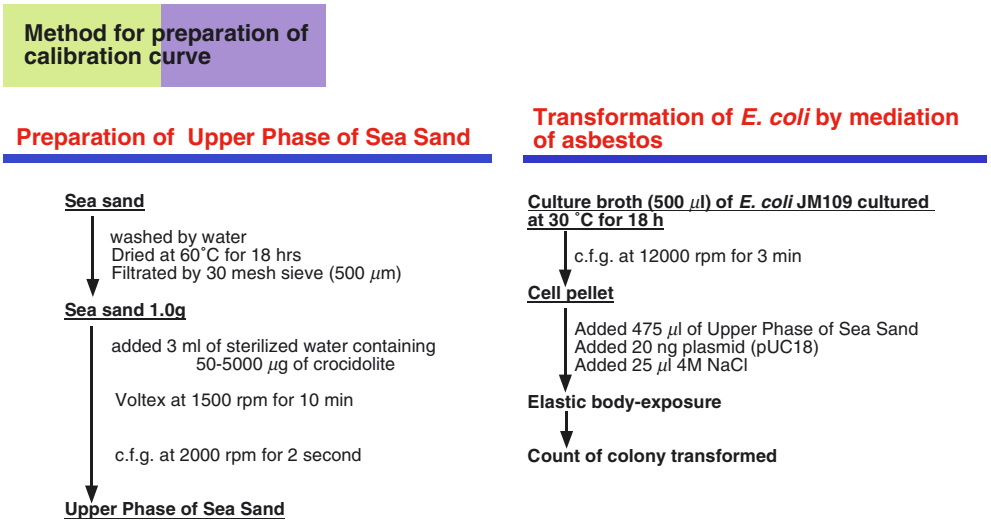

Fig. 3 Method for preparing calibration curve for asbestos quantification. *E. coli* transformants were counted at various concentrations of crocidolite in sea sand

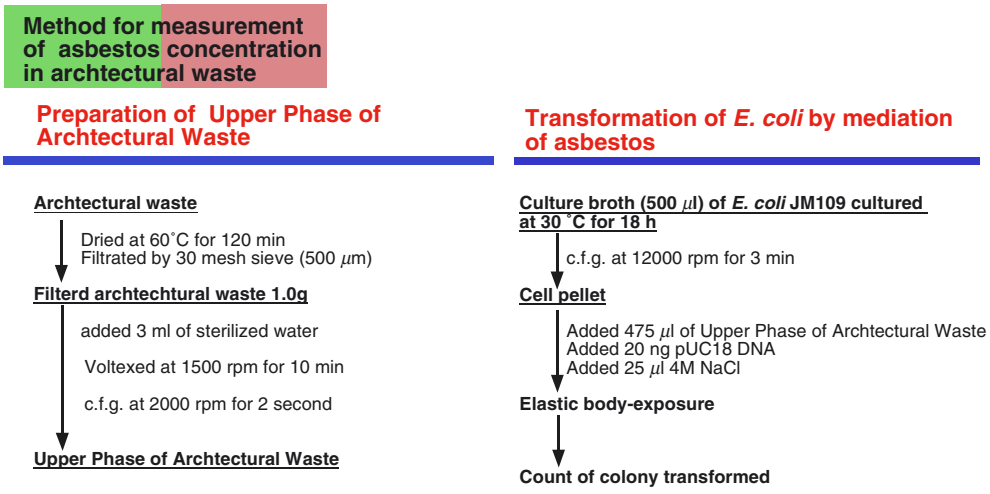

Fig. 4 Proposed method for environmental asbestos quantification. Asbestos determination from gravelly sand obtained from building materials is demonstrated

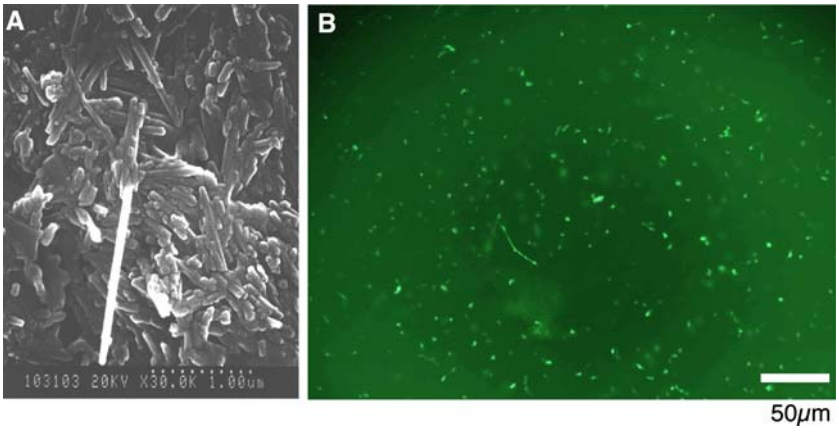

Fig. 5 Electron microscopic visualization of crocidolite in aqueous colloidal solution (**a**). Crocidolite was identified as acicular crystals. Concavoconvex surface was an artifact of spattering. Fluorescence microscopic visualization of crocidolite after adsorbing FITC-conjugated DNA oligomer (**b**). *Bar* 50 μm

of the conventional method. Sensitivity for asbestos detection is sharp and the detection limit is about 100 μg/g asbestos in sea sand.

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