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Liquid-phase Sample Preparation Method for Real-time Monitoring of Airborne Asbestos Fibers by Dual-mode High-throughput Microscopy*

Myoung-Ock Cho, Jung Kyung Kim, Hwataik Han, and Jeonghoon Lee

Abstract— **Asbestos that had been used widely as a construction material is a first-level carcinogen recognized by the World Health Organization. It can be accumulated in body by inhalation causing virulent respiratory diseases including lung cancer. In our previous study, we developed a high-throughput microscopy (HTM) system that can minimize human intervention accompanied by the conventional phase contrast microscopy (PCM) through automated counting of fibrous materials and thus significantly reduce analysis time and labor. Also, we attempted selective detection of chrysotile using DksA protein extracted from *Escherichia coli* through a recombinant protein production technique, and developed a dual-mode HTM (DM-HTM) by upgrading the HTM device. We demonstrated that fluorescently-labeled chrysotile asbestos fibers can be identified and enumerated automatically among other types of asbestos fibers or non-asbestos particles in a high-throughput manner through a newly modified HTM system for both reflection and fluorescence imaging. However there is a limitation to apply DM-HTM to airborne sample with current air collecting method due to the difficulty of applying the protein to dried asbestos sample. Here, we developed a technique for preparing liquid-phase asbestos sample using an impinger normally used to collect odor molecules in the air. It would be possible to improve the feasibility of the dual-mode HTM by integrating a sample preparation unit for making collected asbestos sample dispersed in a solution. The new technique developed for highly sensitive and automated asbestos detection can be a potential alternative to the conventional manual counting method, and it may be applied on site as a fast and reliable environmental monitoring tool.**

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M.-O. Cho was with the Department of Mechanical Engineering, Graduate School, Kookmin University, Seoul 136-702 Korea. She is now with PaSce co. (corresponding author to provide phone: +82-2-910-5409; fax: +82-2-910-4839; e-mail: fallslover@naver.com).

J. K. Kim is with the Department of Family Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria 0084 South Africa, on sabbatical leave from the School of Mechanical Systems Engineering, Kookmin University (e-mail: jkkim@kookmin.ac.kr).

H. Han is with the School of Mechanical Systems Engineering, Kookmin University (e-mail: hhan@kookmin.ac.kr).

J. Lee is with the School of Mechanical Engineering, Korea University of Technology and Education, Cheonan 330-708 Korea (e-mail: jlee@koreathech.ac.kr).

I. Introduction

Asbestos is a naturally occurring tube-shaped silicate mineral with nano size [1]. Asbestos has advantages of durability, heat resistance etc.; for the reason, it has been used various fields as a construction material worldwide. However, now the use of asbestos is ban in most developed countries since it has been known that inhalation of asbestos may cause lung cancer, malignant mesothelioma or other serious respiratory diseases, and many people still die from asbestos related diseases in the world [2].

Phase-contrast microscopy (PCM) is one of the established methods to detect airborne asbestos. Also it is cost-effective and has relatively simple process. Lung cancer or asbestosis has been related with thin asbestos fibers (< 0.25 μm) [3]. However the hardness of detecting thin fibers due to an optical resolution limit and the inability of clear distinction between certain types of asbestos are biggest disadvantages of PCM [4]. The PCM method also produces inaccurate results by subjective opinion of operator. It needs to develop an innovative method for detecting asbestos simply in view of these facts. Recently, automatic image analysis based asbestos detection methods have been studied in many areas. Kawabata *et al.* [5] developed an image based qualitative asbestos detection method, and they tried to distinguish asbestos fibers among other particles using shape information and color dispersion of asbestos fiber. Other study groups also developed automatic image analysis method for detecting asbestos by "image matching" using dispersion staining [6, 7].

We developed an image analysis based asbestos detection method, high-throughput microscopy (HTM), through our previous study and demonstrated that automatic asbestos counting is feasible by comparing results from HTM and PCM [8] methods. The HTM method is still unable to detect asbestos fibers specifically for distinguish certain types of asbestos although it complements many drawbacks of PCM. There was a breakthrough in selective detection and identification of asbestos fibers through adapting a biotechnological approach as reported in Kuroda *et al*. [9] and Ishida *et al*. [1]. Asbestos-adhesive proteins, such as DksA and GatZ, were used in their studies and they could detect chrysotile or amosite and crocidolite, selectively using fluorescence tagging method. All types of asbestos have their own chemical composition, morphology, durability and properties implying that various kinds of asbestos fibers should be treated differently [10]. Selective detection of asbestos using those protein based methods would enable us to cope with the problem.

In this study, we made use of chrysotile-adhesive protein, DksA, in HTM method in order to enhance sensitivity and selectivity in high-throughput manner. DksA is an α-helical protein that is comprised of 151 amino acids, it also binds to RNA polymerase (RNAP) and is associated with controlling rRNA transcription by ppGpp in vivo [11]. We adapted fluorescence-tagging method for visualization of chrysotile asbestos and modified our HTM device to build a dual-mode HTM (DM-HTM) which can acquire both reflected-light images and fluorescence images. We tested the DM-HTM system with chysotile samples and mixed asbestos samples to confirm enhanced sensitivity and selectivity. A stack of reflection and fluorescence images were analyzed automatically and total fiber counts were compared with those from manual counting by a human researcher. While the fluorescence tagging method improved the sensitivity and selectivity of asbestos detection substantially, it is yet to be applied to the sample preparation procedure for detecting airborne asbestos. Thus we also attempted to develop a liquid-phase asbestos sample preparation method to apply our DM-HTM method directly to asbestos in air.

II. Material and Method

A. Configuration of dual-mode HTM

We modified the HTM device we developed previously to detect fluorescence signal. Two linear stages (M-426A; Newport) and two linear actuators were used in order to make a movement perpendicularly. An objective lens (NT36-132; Edmund Optics) was equipped at the end of a 160-mm scope tube, and a charge-coupled device (CCD) camera (IMB-20FT; imi tech) was fastened on the top of the tube. Optical filters were added in the middle of the optical pathway for fluorescence imaging, which also reduced the level of autofluorescence in reflection imaging. An emission filter (ET605/70m; Chroma) and a dichroic mirror (T565lpxr; Chroma) were equipped at the boundary of the CCD camera and the scope tube, and an excitation filter (ET545/25x; Chroma) was inserted in front of a green LED (TouchBright X-3; Live Cell Instrument) which is a fluorescent light source. The green light passed the excitation filter and excited the fluorescent dye bound to the asbestos, and the fluorescence signal passed the emission filter was detected by the CCD camera. Fig. 1 shows a schematic of our dual-mode HTM device. We controlled the stages automatically using an application software (Zaber Console, Zaber Technologies), and also used a trigger system in order to obtain images in regular interval. The stages moved every 1 second, and images were saved after 0.5 s at the end of the motion of the stages. The actuators connected to the stages sent a signal to a signal control box (Board Lab Inc.) and the 2V signal triggered the CCD camera after having been amplified into TTL signal through the control box. The travel distance of the stages was adjusted according to the aspect length of a display window.

B. Preparation of asbestos-adhesive recombinant protein

Genomic DNA was extracted from *Escherichia coli* (*E. coli*) and the polymerase chain reaction (PCR) was followed to amplify the template using the primer set that we engineered refer to the study by Kuroda *et al*. [9]. Plasmid pET21-α and the amplified DNA were digested using NheI and BamHI, respectively, and the DNA fragments were

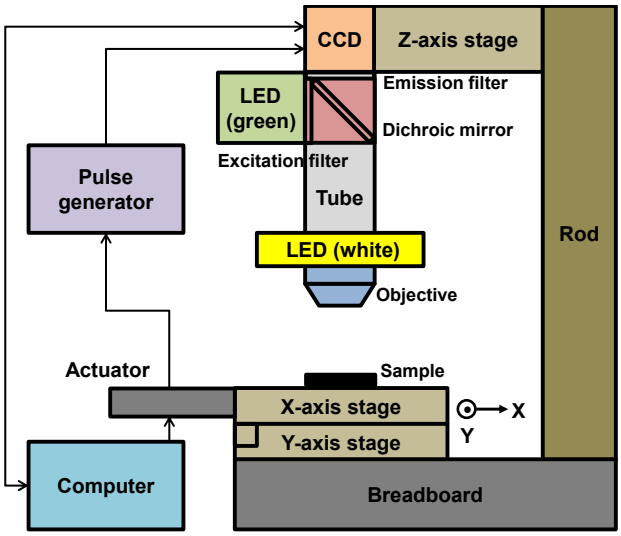


Figure 1. A schematic of dual-mode HTM for both reflection and fluorescence imaging

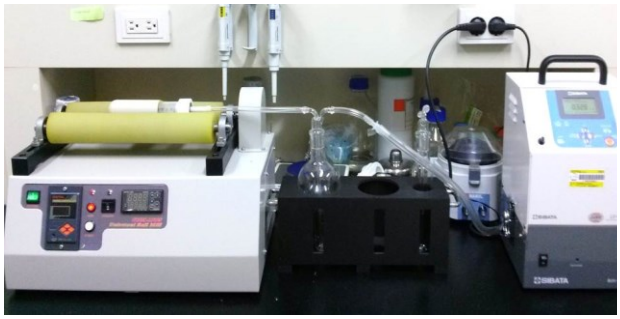


Figure 2. Experimental setup for liquid-phase asbestos sample preparation using conventional filter method.

inserted between the NheI and BamHI sites of the plasmid. The vectors including the DNA fragments were incubated for 16 h at 36 °C on a Luria-Bertani (LB) agar plate after transformed into competent cells, DH5-α. DNA sequencing was conducted using the recombinant DNA extracted from the bacterial colony on the agar plate. Then the recombinant DNA was retransformed into BL21 (DE3) cells, spread on LB agar plates and incubated for 16 h at 36°C. Colonies formed on the agar plates were cultured in LB medium until it reaches 0.4 at OD_{600}, then 0.1 mM of IPTG was added. The culture was incubated for additional 3 h at 37°C, 180 rpm. The transformed *E. coli* lysate was purified using Ni-NTA His-tag affinity chromatography column (Clontech) and dialyzed using a commercial kit (20-kD Slide-A-Lyzer Dialysis Cassette; Thermo Scientific), after lysing process by a microfluidizer (M-110P, Microfluidics).

C. Experimental setup for liquid-phase asbestos sample preparation

We attempted to develop a technique for making liquid phase asbestos sample using an impinger that is used to collect odor molecules in the air. Air sampler, impinger and the sample tube that we fabricated for this work were

connected by thin rubber tubes. We tried to confirm this system using 30 μm PMMA beads. Beads were collected in impinger bottle containing 5 mL of water at a flow rate of 10 L/min for 30 min, and the collected beads in the impinger were calculated as volumetric concentration. The control sample was prepared in a tube with 5 mL of water and the same amount of beads. The beads counting result using the impinger system was compared with the result from control sample. We also tried conventional filter method using this system by locating the filter between the air sampler and the sample tube, instead of the impinger bottle. Fig. 2 shows our experimental setup for preparing liquid-phase asbestos sample.

III. Result and Discussion

A. *Fluorescence imaging of chrysotile by dual-mode HTM*

The dual-mode HTM includes an additional light source and fluorescence filters and it enables visualization of chrysotile with the fluorescence-tagged protein. We verified the property of the protein by fluorescence imaging using the modified HTM device. We prepared mixed asbestos sample formed with chrysotile and amosite in order to confirm whether the protein attaches only to chrysotile in interference of amosite. The scan pathway was changed so that the stages get back to where it started, and it was possible to obtain the reflected light images and fluorescence images at the same point. Fig. 3 shows the images of mixed asbestos sample taken in both reflected light and fluorescence modes. The concentrations of chrysotile and amosite were varied therefore chrysotile concentration was reduced in high concentration of amosite within the mixed asbestos sample. Although it is hard to detect chrysotile in high concentration of amosite solution from reflection images, the fluorescence imaging mode offers clear image of chrysotile with greatly reduced background.

B. *Selective and high-throughput detection of asbestos*

Both reflection and fluorescence images were automatically analyzed using an image analysis program, ImageJ, and the counting results were compared against the actual concentration of chrysotile samples. Since chrysotile was serially diluted with amosite sample solution, the concentration of chrysotile increased with decreasing amosite concentration. The result from the automatic counting of the mixed asbestos samples against chrysotile concentration was shown in Fig. 4(a). As the amosite sample contains more small fibers than the chrysotile at the same weight, the entire number of fibers is reduced at high chrysotile concentration of the mixed sample. However the total number of chrysotile fibers is proportionally increased in fluorescence imaging mode which enables to detect only chrysotile fibers excluding other asbestos and background noise. Fig. 4(b) shows automatic counting results from analyses of both fluorescence and reflection images with respect to chrytotile concentration. The fiber counts from reflection image analysis tend to be overestimated when the slide sample includes air bubbles, despite the fact that uneven and noisy background are significantly reduced by our improved adaptive image processing algorithm with variable parameters. In contrast, automatic fiber counts obtained from fluorescence image analysis were linearly proportional to the actual concentration of asbestos samples, confirming that the sensitivity and the

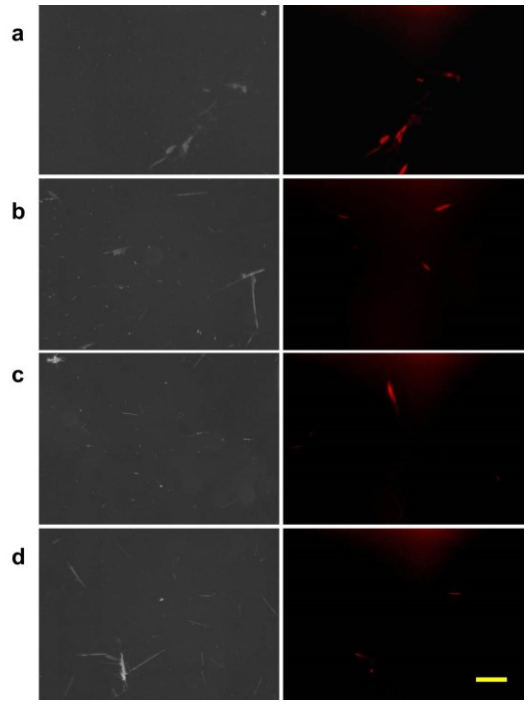
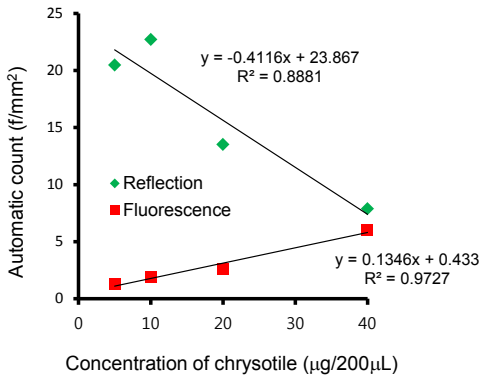
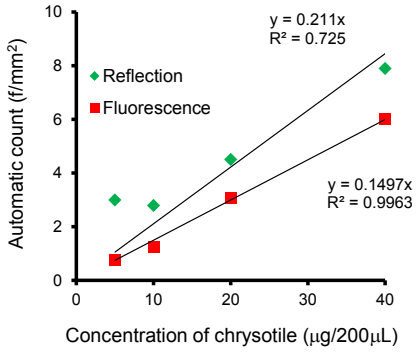


Figure 3. Reflection and fluorescence images of mixed samples of chrysotile with amosite in different concentrations. Reflection images (left), fluorescence images (right). Concentrations of chrysotile: (a) 200, (b) 100, (c) 50, (d) 25 μg/mL. Bar = 100 μm.



(a)



(b)

Figure 4. Asbestos counting results from analyses of reflection and fluorescence images. (a) Automatic counts of the mixed asbestos samples against chrysotile concentrations. (b) Chrysotile fiber counts obtained by HTM and manual counting methods.

TABLE I. BEAD COUNTING RESULT USING LIQUID-PHASE SAMPLE PREPARATION SYSTEM

	Control sample	Liquid sample	Filter	Washed sample
N/image	41.1	1.719	29.9	16.7
Total($\times 10^4$)	1394	58.2	3.62	567
N/cc		1.941	0.1206	

selectivity of HTM are enhanced by adapting fluorescence imaging and asbestos-adhesive proteins. It was reported that fluorescently-labeled asbestos fibers as thin as 30 ~ 35 nm can be detected by fluorescence microscopy [12].

C. *Testing of liquid-phase asbestos sample preparation system*

We tried the experiment for liquid-phase asbestos sample preparation in order to develop a one-step system that is able to collect and selectively detect the airborne asbestos using fluorescence-tagged protein, simultaneously. PMMA beads with 30-μm diameter were used before using real asbestos sample. A sample tube including 70 mg of microbeads was kept spinning on a milling machine to induce dispersion of the beads. A filter was inserted at the bottom of the sample tube to prevent low pressure and to induce the air circulation within the tube when the air sampler absorbs the air. The sample tube was connected to the impinge bottle including 5 mL of water inside, and also connected with the air sampler. Therefore microbeads in the sample tube were mixed with the water inside the impinge bottle through the air pathway. The liquid-state beads sample collected in the impinge bottle was prepared to make a sample slide after 30 min. Image acquisition and analysis were conducted using the DM-HTM system, and the results were compared with a control sample that was mixed with 5 mL water and the same amount of beads. As shown in Table I, the number of beads that were included in the liquid-phase sample was approximately a quarter of the control sample. The beads remaining in the sample tube was diluted in water, and analyzed to find main cause of the loss. As a result, combined number of microbeads from the liquid sample and in the sample tube was only a half of the control sample, indicating that the amount of beads remaining in the tubing and inside wall of the impinge bottle is not negligible. It still remains as a further study to find the way for reducing those losses in order to complete the system for a practical application.

IV. CONCLUSION

In this study, we improved the selectivity in asbestos detection by using the chrysotile-binding protein DksA, and also developed a liquid-phase asbestos sample preparation method in order to enhance feasibility of the dual-mode HTM system. The asbestos-adhesive protein was extracted from *E. coli* using a recombinant protein production method, and its selectivity for chrysotile asbestos was proved through several experiments analyzing samples that included other particles and mixed with different kinds of asbestos. We used a modified version of the HTM device developed in our previous study, and verified another application by using it for the fast counting of chrysotile fibers. However, a new sampling or pretreatment method should be established in order to apply the protein to the detection of on-site airborne asbestos. The method we tried in this study for preparing liquid-phase asbestos sample collected in air still has some problems such as big loss during operation. This highly selective detection method, combined with other known biological techniques and high-throughput microscopy, will become a fast and reliable environmental monitoring tool.

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