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Review

Rapid on-site detection of airborne asbestos fibers and potentially hazardous nanomaterials using fluorescence microscopy-based biosensing

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A large number of peptides with binding affinity to various inorganic materials have been identified and used as linkers, catalysts, and building blocks in nanotechnology and nanobiotechnology. However, there have been few applications of material-binding peptides in the fluorescence microscopy-based biosensing (FM method) of environmental pollutants. A notable exception is the application of the FM method for the detection of asbestos, a dangerous industrial toxin that is still widely used in many developing countries. This review details the selection and isolation of asbestos-binding proteins and peptides with sufficient specificity to distinguish asbestos from a large variety of safer fibrous materials used as asbestos substitutes. High sensitivity to nanoscale asbestos fibers (30–35 nm in diameter) invisible under conventional phase contrast microscopy can be achieved. The FM method is the basis for developing an automated system for asbestos biosensing that can be used for on-site testing with a portable fluorescence microscope. In the future, the FM method could also become a useful tool for detecting other potentially hazardous nanomaterials in the environment.

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1 Introduction

Over the past two decades, a large number of peptides binding to various inorganic materials such as metals, metal oxides, metal compounds, carbon materials, and semiconductors have been selected using combinatorial display systems [1–8]. These binding peptides have been used as molecular linkers to control the assembly of proteins on inorganic material surfaces [1–3], catalysts to synthesize nanoparticles [4, 5], and building blocks or templates to assist the co-assembly of different materials for nanotechnology applications [6–8]. Fluorescent labeling of material-binding peptides should make it possible to apply fluorescence microscopy (FM) for the detection and measurement of nanomaterials. FM is an extremely selective and sensitive technique that has numerous advantages over other types of optical and even electron microscopy [9]. The key advantage of FM in the field of environmental biosensing is the ability to distinguish and selectively visualize target materials. At present, there are relatively few examples of successful application of material-binding peptides in the field of biosensing.

This review therefore focuses on a rare success story – the application of material-binding proteins and peptides for the detection of asbestos, a dangerous industrial toxin that is still widely used in many developing countries [10, 11]. Compared with testing for other industrial pollutants, common analytical methods for airborne asbestos fibers suffer from several major shortcomings. First, asbestos analysis requires a careful examination of

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Abbreviations: **EDX**, energy dispersive X-ray spectroscopy; **FM**, fluorescence microscopy; **LED**, light emitting diode; **NIOSH**, US national institute for occupational safety and health; **NMR**, nuclear magnetic resonance; **PCM**, phase contrast microscopy; **ROS**, reactive oxygen species; **SEM**, scanning electron microscopy; **TEM**, transmission electron microscopy; **WHO**, World Health Organization

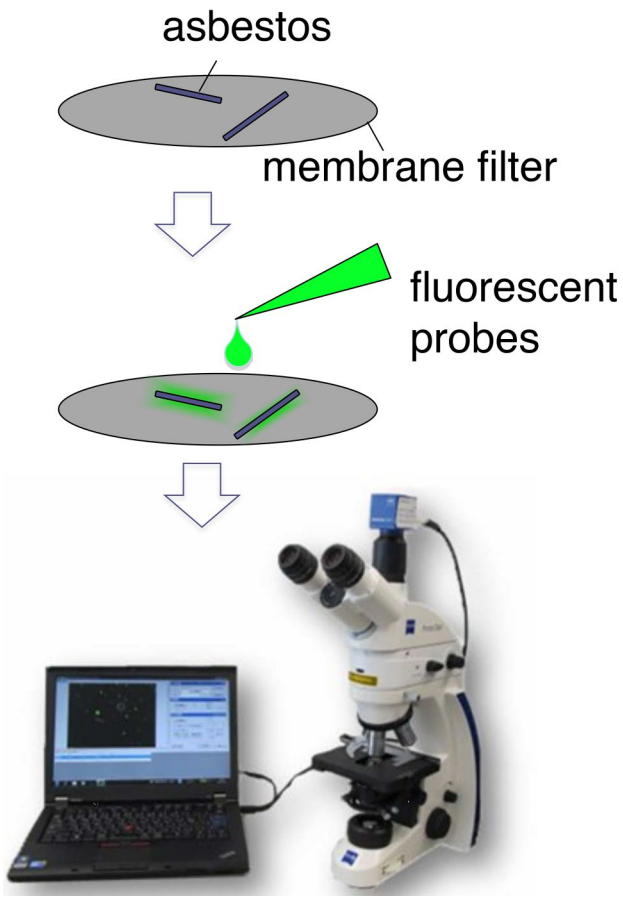

Figure 1. Simplified scheme of asbestos detection using fluorescent probes. Airborne asbestos fibers are trapped on a filter membrane by filtering ambient air. After prewetting the membrane, an assay buffer containing fluorescent-labeled asbestos-binding proteins [27, 45] is applied, followed by washing with a washing buffer. The samples are observed under a fluorescence microscope (Primo Star iLED, Carl Zeiss Microscopy, Oberkochen, Germany) equipped with an AxioCam ICm1 CCD (Carl Zeiss Microscopy). Automated fiber counting is conducted using asbestos counting software [49] installed on a notebook computer running the Windows 7 operating system.

a filter membrane under optical microscopy to count fibers and/or electron microscopy and X-ray analysis to identify and distinguish asbestos from a large variety of safer fibrous materials used as asbestos substitutes. The analysis is extremely labor intensive because the toxicity of airborne asbestos fibers depends not on the total amount (weight) of the inhaled asbestos but on the number of fibers with specific dimensions, which must be manually identified and counted. Second, optical microscopy methods cannot detect the thinnest (nanoscale) asbestos fibers, which are highly toxic. Third, subjective errors are common during asbestos identification and counting, which relies on a complicated set of counting rules. Human error can result in considerable variability between test results obtained by different analysts or laboratories [12]. Finally, due to the lack of portable instrumentation, airborne dust samples that are collected in the field frequently need to be sent to remote laboratories, resulting in long testing times. There are likely few analytical methods that, despite such shortcomings, have remained largely unchanged over the past few decades.

Can asbestos testing be made faster, more reliable and less demanding in terms of analysts' effort and skill? We believe that it can, thanks to the development of a novel FM-based biosensing method (FM method, Fig. 1) using fluorescent-labeled binding peptides with sufficient specificity to distinguish asbestos from non-asbestos fibers and high sensitivity for nanoscale asbestos fibers. Because the fluorescent probes do not stain non-asbestos fibers, the FM method can simplify automated counting of asbestos fibers by image analysis software. With the automated fiber counting, asbestos testing would no longer require any special skills beyond basic microscope operation. Furthermore, the development of portable fluorescence microscopes would allow for rapid on-site testing [13] instead of sending samples to specialized asbestos laboratories. This paper reviews the steps towards developing a rapid on-site testing system for asbestos fibers. It focuses on two aspects of asbestos detection that will likely prove crucial for future biosensing applications in the field of nanotechnology: the selection of material-binding peptides and their application in the FM method.

2 Asbestos and asbestos analytical methods

2.1 Asbestos problems

Asbestos fibers possess a number of useful properties, such as high ultimate tensile strength, low thermal conduction and relative resistance to chemical attack. Asbestos has been widely used in construction materials, including insulation board, asbestos cement, floor tiles and water supply lines, as well as in a number of other products such as fire blankets, clutches, gaskets, brake linings and brake pads for automobiles (http://www.who.int/mediacentre/factsheets/fs343/en/). Because asbestos is composed of microscopic bundles of silicate fibers, asbestos fibers can become airborne when asbestos-containing materials are damaged or disturbed. Exposure to airborne asbestos fibers causes lung cancer and pleural mesothelioma [14, 15]. According to a recent WHO report, approximately 125 million people are exposed to asbestos in the workplace, and at least 107 000 people die each year from asbestos-related disease (http://www.who.int/mediacentre/factsheets/fs343/en/). Although the use of asbestos is now prohibited in most developed countries, large amounts of asbestos-containing materials still remain in many older buildings. In Japan, the amount of such materials is estimated to be approximately 40 million tons (http://worldasbestosreport.org/articles/killing_future/great_hanshin.php). Renovation or demolition of

older buildings can frequently trigger the release of airborne asbestos fibers into the environment. Asbestos contamination therefore remains a major problem, with many countries reporting a rising incidence of asbestos-linked lung cancers and pleural mesothelioma [11, 14, 15].

2.2 Shortcomings of asbestos analytical methods

Managing the risks associated with exposure to asbestos should involve the measurement of airborne asbestos. To measure the concentration of airborne asbestos fibers, ambient air is filtered through a membrane filter. After the filter is cleared with acetone vapor, fibers that satisfy the standard definition by WHO (fibers longer than 5 μm and thinner than 3 μm and with aspect ratios larger than 3:1) are counted under optical phase contrast microscopy (PCM) (Asbestos and other fibers by PCM: Method 7400, in: *National Institute of Occupational Safety and Health [NIOSH] Manual of Analytical Methods, 2nd issue*, Washington, DC 1994). While simple and cheap, PCM has a number of limitations. It is nearly impossible to detect very thin asbestos fibers (less than 0.25 μm in diameter), which are abundant in chrysotile asbestos, due to the diffraction limit [12]. In practice, the detection limit may be even higher because of sample contamination (particularly the presence of dust particles). Epidemiological studies have suggested that both lung cancer and asbestosis are most strongly associated with exposure to thin fibers (<0.25 μm) [16], whereas mesothelioma is associated with exposure to fibers thinner than approximately 0.1 μm [17]. PCM analysis may therefore seriously underestimate asbestos-related risks. Furthermore, the use of non-asbestos fibers in the construction industry as safer substitutes for asbestos has complicated the asbestos analysis because PCM test cannot distinguish these fibers from asbestos fibers. In Japan, all PCM fiber counts above 1 f/L require re-testing using scanning electron microscopy (SEM) or transmission electron microscopy (TEM) with energy dispersive X-ray spectroscopy (EDX) to identify asbestos fibers ((Asbestos monitoring manual [in Japanese], Ministry of the Environment of Japan, 2010, http://www.env.go.jp/press/files/jp/15810.pdf). In the US, the TEM-based method is recommended "if serious contamination from non-asbestos fibers occurs in samples" (Asbestos and other fibers by PCM: Method 7400, in: *National Institute of Occupational Safety and Health [NIOSH] Manual of Analytical Methods, 2nd issue*, Washington, DC 1994). However, wider use of electron microscopy would sharply increase the cost and duration of asbestos analysis, making comprehensive asbestos monitoring impractical in many occupational settings. A rather limited alternative to electron microscopy is polarized light microscopy, which may be used to identify and eliminate interfering non-crystalline fibers with diameters >1 μm (Asbestos and other fibers by PCM: Method 7400, in: *National Institute of Occupational Safety and Health [NIOSH] Manual of Analytical Methods, 2nd issue*, Washington, DC 1994). The NIOSH has made the development of improved analytical methods for asbestos fibers a strategic research goal [12].

2.3 A new approach to asbestos detection using material-binding peptides

FM is one of the most important analytical tools used in modern life science, with sufficient sensitivity for detecting single molecules [9]. An immunofluorescence technique using a fluorescent-labeled antibody allows for the visualization of the distribution and interaction of specific biomolecules within a cell. In one of the pioneering studies on the application of FM to inorganic materials, Whaley et al. demonstrated the visualization of very thin gallium arsenide (GaAs) lines on a GaAs/SiO_2-patterned substrate under FM using a GaAs-binding peptide probe [18]. The preferential attachment of fluorescent-labeled material-binding peptides to a surface with a specific chemical and structural composition enables the selective visualization of a target material under FM. By applying FM to the detection of asbestos, it should be possible to overcome the shortcomings of the conventional PCM method, such as the inability to distinguish asbestos from non-asbestos fibers, which is currently compensated for by electron microscopy and EDX analysis, and low sensitivity to very thin fibers. The successful application of FM requires the development of a probe that has (i) sufficient binding affinity to visualize extremely thin asbestos fibers under FM and (ii) high specificity to distinguish non-asbestos fibers, particularly those widely used for construction, from asbestos fibers.

3 Material-binding peptides

3.1 Screening of material-binding peptides

The selection of material-binding peptides was first demonstrated using a cell-surface display method [19, 20]. Since then, random peptide libraries of the more convenient phage display method have been utilized for the selection of binding peptides for a number of inorganic materials. To date, a large number of peptides binding to metals, metal oxides, metal compounds, polymers, carbon materials, and semiconductors have been identified [1–8]. Furthermore, advances in antibody engineering have enabled the fabrication of a number of antibodies specific to nonbiological organic molecules [21], metal ions [22], and the surfaces of organic materials [23]. Watanabe and coworkers identified an antibody fragment that preferentially binds to gold over platinum, palladium, and silver from a phage-displayed library of the variable region of heavy and light Fv chains of human antibodies [24].

Although phage display is the most popular method for selecting material-binding peptides, it has several limitations. For example, this method does not guarantee the affinity of the selected peptides to the target materials. In some cases, a peptide selected by phage display fails to bind to its target when detached from the phage. A synergistic effect on the affinity mediated by the interaction between the peptide and phage body can sometimes contribute to the binding [25]. Another shortcoming of the method is that some of the phage proteins may have intrinsic affinity to certain inorganic materials, leading to nonspecific binding of random phages during the selection process and, therefore, failure to select specific binding peptides.

As an alternative method that is free of the above mentioned shortcomings, we [26–28] and another group [3, 29–31] used cellular protein libraries to select material-binding proteins. In this method, a target material was first mixed with a protein mixture of bacterial cell lysate. Proteins that preferentially adsorbed onto the target material were co-precipitated and identified by a peptide-mass-fingerprinting method. Minimal binding domains or peptide sequences responsible for the material-binding properties were identified using a deletion mutant library of the binding protein [26]. In another study, binding peptides were directly selected from a peptide library prepared by trypsin digestion of the binding protein [3]. Kumada et al. successfully isolated peptides binding to the surface of hydrophilic polystyrene [29], polycarbonate [30], poly(methylmethacrylate) [30], and silicon nitride (SiN) [31] from the amino acid sequences of *E. coli* proteins.

Although most material-binding proteins and peptides have been selected for a particular purpose, some have been used in a number of applications. A good example is *E. coli* ribosomal protein L2, which was identified as a silicon-binding protein (Si-tag) using silicon particles as a target material [26]. Because the surface of silicon particles is oxidized, Si-tag presumably binds to silicon dioxide [32]. Si-tagged protein A was used for oriented immobilization of antibodies on the surface of a silicon wafer [33] and recently on a silicon ring resonator to develop an immuno-biosensor [34, 35]. Si-tag could also be used as a protein tag for affinity purification using silica particles [36, 37].

3.2 Binding specificity of peptides to inorganic materials

In many biosensing applications, including asbestos detection, there is a need to distinguish toxic substances from similar but harmless materials. This task requires high binding specificity, yet whether such specificity could be obtained for most inorganic materials is still unclear because inorganic materials have simpler and rather homogeneous atomic structures compared with those of bio-macromolecules. However, high binding selectivity has been reported for several peptides. Whaley et al. selected peptides that bind to GaAs but not to SiO_2 from phage display libraries [18]. Interestingly, one of the clones preferentially binds to GaAs(100), which was used for its selection, over the different crystalline faces of GaAs, (111)A (gallium terminated) or (111)B (arsenic terminated). The gold-binding peptide GBP1, which was originally selected from a cell-surface-display library, specifically binds to gold over platinum and silicon dioxide [6]. A phage expressing titanium-binding peptide TBP-1 was found to bind to silver and silicon, although it did not bind to gold, chrome, platinum, tin, zinc, copper or iron [38]. In our experience, the selected peptides can generally bind to several inorganic materials but can sometimes be made to distinguish a particular material under carefully chosen buffer conditions.

Both the affinity and specificity of binding peptides stem from chemical (hydrogen, hydrophobic and electrostatic bonding) and structural (size and morphology) recognition mechanisms that ultimately depend on peptide sequence. The sequences of many types of material-binding peptides have been listed in previous reviews [1, 4, 6]. Similar tetrapeptide repeats (SEKL and GASL) have been observed in the peptides that control the morphology of gold crystals [1]. Amino acids commonly found in material-binding peptide sequences, which might play an important role in the binding, have been reported [1].

Analysis of molecular structures of material-binding peptides is important to understand the mechanism of specific binding. To date, several NMR studies have been performed to understand the molecular basis of peptide binding to inorganic surfaces [39–43]. These studies have examined the adsorption of salivary proteins on hydroxyapatite [39, 40], calcium carbonate-bound polypeptides from mollusk shells [41], and a gold-binding peptide selected from a combinatorial library [42]. Recently, Mirau et al. determined the structure of three related peptides bound to silica and titania nanoparticles using the solution NMR method [43]. These peptides have been found to have a similar structure, suggesting that a common structural motif is involved in their ability to recognize silica and titania surfaces [43].

4 Fluorescence imaging of asbestos fibers

4.1 Development of asbestos-binding fluorescent probes

Asbestos consists of two mineral groups, serpentine and amphibole, which differ in their crystal structure and toxicity [12]. Chrysotile ($Mg_6Si_4O_{10}[OH]_8$), which accounts for 90% of world asbestos production, is the only type of asbestos that belongs to the serpentine group [12]. The surface of these fibers is magnesium-rich and positively

charged under physiological conditions. Amphibole group is composed of five types of asbestos, amosite ($[Fe, Mg]_7Si_8O_{22}(OH)_2$), crocidolite ($Na_2[Fe^{3+}])_2[Fe^{2+}]_3Si_8O_{22}[OH]_2$), tremolite ($Ca_2Mg_5Si_8O_{22}[OH]_2$), actinolite ($Ca_2[Mg, Fe]_5Si_8O_{22}[OH]_2$), and anthophyllite ($[Mg, Fe]_7Si_8O_{22}[OH]_2$). The surface of amphibole fibers is predominantly composed of crystalline silica (long ribbons of silicon tetrahedra) and is negatively charged.

Because of the different properties of chrysotile and amphibole asbestos, at least two different probes were necessary to detect all types of asbestos. The first asbestos probe, DksA, was selected as a binding protein with high affinity to chrysotile asbestos from the *E. coli* cellular protein library [27]. The dissociation constant (K_d) of DksA to chrysotile was determined to be approximately 3.5 nM by Scatchard analysis [27]. To evaluate the specificity of fluorescent-labeled DksA, binding tests were performed for chrysotile and all the remaining types of asbestos, as well as ten types of common non-asbestos fibrous materials provided by the Japan Fibrous Material Research Association [44]. The non-asbestos materials tested included nearly all asbestos substitutes commonly used in the construction industry as well as several fiber types used in other industries. The fluorescent-labeled DksA bound to chrysotile but neither to amphibole asbestos nor to other non-asbestos fibers, indicating highly selective binding of the probe to chrysotile asbestos.

Because the five types of amphibole asbestos share a similar crystal structure [12], amosite asbestos was used for screening and for the development of a cross-reactive amphibole asbestos probe. Proteins (GatZ, H-NS) were selected from the *E. coli* cellular protein library as amosite-binding proteins. However, these proteins also bound to asbestos substitutes such as the natural fibrous mineral wollastonite ($CaSiO_3$) [28, 45]. A minimal binding peptide (31 amino acids) responsible for the amosite-binding property was identified using the H-NS protein deletion library [45]. The amosite-binding peptide showed a higher specificity than that of the original H-NS protein but unfortunately a lower affinity, suggesting the presence of multiple binding sequences with affinity to various types of fibers. It has been reported that the repetition of material-binding sites (peptide sequence) can increase binding affinity [1, 20, 24], and the multiple display of a binding peptide on a ferritin was observed to notably increase the affinity compared with that of the single peptide [25]. In our research, a commercially available fluorescent-labeled streptavidin tetramer was used as a scaffold to display four molecules of the biotin-labeled amosite-binding peptide [45]. The affinity of the streptavidin tetramer complex was approximately 250-fold higher than that of the single amosite-binding peptide [45]. The K_d of the complex for amosite was 1.0 nM [45]. An alanine-scanning experiment indicated that three lysine residues in the peptide were primarily responsible for the binding to amosite, suggesting that the probable binding mechanism is electrostatic interaction [45]. The streptavidin tetramer complex had sufficient affinity and specificity for detecting all five types of asbestos in the amphibole group and could be used to distinguish them from ten types of commonly used non-asbestos fibrous materials, including wollastonite [45]. The multiple fluorescent labeling of different probes can be used to identify chrysotile and amphibole asbestos simultaneously in a single sample (Fig. 2).

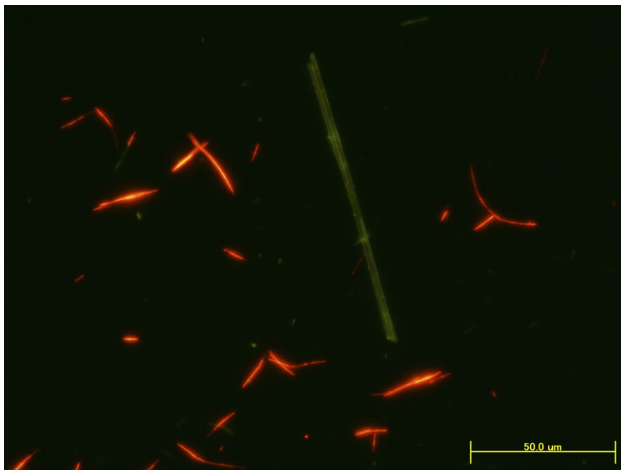

Figure 2. Fluorescence image of asbestos using multiple fluorescent labeling. DksA-Cy3 (red) [27] and GatZ-fluorescein (green) [28] proteins were used for fluorescence staining of chrysotile and amosite, respectively. The fluorescence image was obtained using a fluorescence microscope (Olympus BX60, Tokyo, Japan) with an Omega XF52 double pass filter (Ex: 490–550 nm, Em: 520–580 nm) and a 40× objective lens (Olympus UplanFl N 40×/0.75 NA Ph2).

4.2 FM method offers comparable sensitivity to SEM

At present, because the commonly used PCM method cannot reliably detect the most toxic fibers (with diameters < 0.25 µm for lung cancer and asbestosis and <0.1 µm for mesothelioma) [16], either SEM or TEM analysis would be necessary to estimate the number of thin fibers. We expected that the FM method would offer higher sensitivity than the PCM method. Very thin chrysotile fibers were examined under PCM and FM in the same field of view (Fig. 3A and 3B). The FM image was clearer than that obtained under PCM, and some of the fluorescent fibers were invisible under PCM. Fig. 3C and 3D as well as Fig. 3E and 3F compare the same region observed under FM and SEM using correlative microscopy. The correlative microscopy relies on a specially designed sample holder that is shared under FM and SEM, a motorized stage, and automated alignment in order to examine the same field of view under FM and SEM platforms [46]. The apparent diameter of the thinnest fibers visible under FM was approximately 30–35 nm as estimated by SEM (Fig. 3F), which is similar to the reported dimensions of

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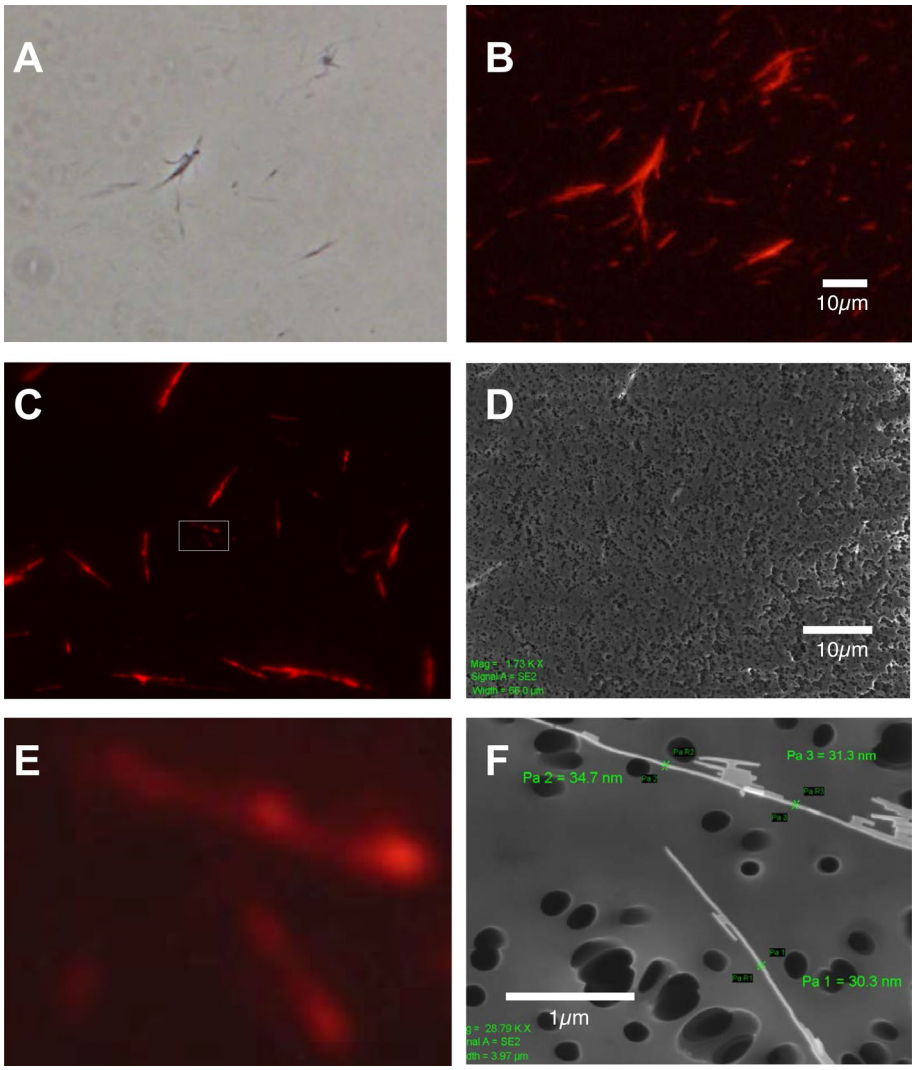

Figure 3. Comparison of fiber visibility on PCM, FM, and SEM micrographs. The PCM and FM micrographs were captured at 500× magnification. The digitally magnified PCM (**A**) and FM (**B**) micrographs are compared for the same field of view. The digitally magnified FM (**C**) and SEM micrographs captured at 1700× magnification (**D**) are compared for the same field of view. The rectangular area in (**C**) is digitally magnified (**E**) to adjust to the corresponding regions of the SEM images at 28 800× magnification (**F**). Micrographs (**C** to **F**) are reproduced from the reference [47]. Image size and resolution were adjusted in Adobe Photoshop.

single chrysotile fibrils [47]. This result indicates that the sensitivity of FM is sufficient to detect single chrysotile fibrils.

The FM method offers the superior fiber visibility at 400× to 500× magnification, which is traditionally used in asbestos analysis. On the other hand, it was difficult to detect the thinnest chrysotile fibers under SEM at 1700× magnification because of the granular background of the membrane filter (Fig. 3D). In our experience, SEM at magnification levels above 4000× is necessary for reliable identification of the thinnest chrysotile fibers, suggesting that 100 SEM micrographs are required to cover a single FM field of view. Moreover, SEM analysis requires EDX for the differentiation of asbestos from non-asbestos fibers. The EDX analysis is extremely labor intensive if several hundred fibers are subject to identification. FM only counts fluorescent asbestos fibers, reducing the testing time and cost. However, it should be noted that because the resolution of FM is limited by optical diffraction, thin fibers appear thicker under FM than under SEM (Fig. 3E and 3F). Despite this shortcoming, the FM method enables rapid, selective, and sensitive counting of asbestos fibers at a fraction of the cost of SEM.

4.3 Development of an automated asbestos counting system

The counting of asbestos fibers must follow a complicated set of counting rules, and there is a need to carefully examine at least 100 fields of view, or to count at least 200 fibers. A large variability between asbestos counts obtained by different analysts and laboratories would be expected because of differences in analyst skill and the degree of adherence to the counting rules (Asbestos and other fibers by PCM: Method 7400, in: *National Institute of Occupational Safety and Health [NIOSH] Manual of Analytical Methods, 2nd issue*, Washington, DC 1994). Measures taken to improve the reliability of asbestos test-

Figure 4. Development of portable fluorescence microscope for on-site detection of asbestos. The compact and portable fluorescence microscope was designed by a Hiroshima University spin-off venture Siliconbio Inc. (Higashi-Hiroshima, Japan) in collaboration with Opto Science Inc. (Tokyo, Japan). The microscope contains a blue LED excitation light source (470 nm, 200 mW) with a constant current output driver, a 470-nm narrow band excitation filter (470QM40, Omega), a dichroic mirror with cutoff at 505 nm (505DRLP, Omega), and a 20× objective lens (*NA* = 0.40, f = 3.32 mm) with a 510 nm long-pass fluorescence filter (510QMLP, Omega). The device does not have a dedicated CCD unit, relying instead on an iPad camera module. A demonstration video is available at the following website: http://siliconbio.co.jp.

ing, such as additional training or participation in inter-laboratory quality control programs, can also contribute to the high cost of asbestos testing. All the currently used methods of asbestos analysis, including FM, suffer from this shortcoming.

A possible solution to these problems is to automate fiber identification and counting using image analysis software. This approach would drastically reduce the cost while increasing the reliability of asbestos testing. The first largely FM-based automated fiber counting system was reported in 2013 by Cho and coworkers [48]. This system relies on a dual-mode high-throughput microscopy (DM-HTM) device that is capable of automated image acquisition. The system was tested using artificially prepared dust-free samples of chrysotile asbestos and showed a good correlation between manual and automated counts. However, Cho and coworkers did not publish any test results for field samples, and the counting software they used (ImageJ freeware) did not implement the rules for counting splayed or crossed fibers. Counting fibers on the field samples is a much more difficult task for any software because the presence of autofluorescent dust particles could considerably reduce the accuracy of fiber identification.

To enable automated fiber counting on field samples, we developed dedicated asbestos counting software with algorithms for counting crossed as well as splayed fibers according to the counting rules [49]. In field samples, long fibers often appear to be split into shorter fragments by overlapping dust particles. To avoid counting these fragments as separate fibers, the software measures the angle between fibers that are adjacent to the same particle and counts them as a single fiber when this angle is within the range of 160 to 200 degrees. Figure 1 shows the software-assisted asbestos counting system composed of an LED fluorescence microscope connected to a notebook computer with a Windows operating system [49]. The identified asbestos fibers are automatically highlighted and numbered by the software. The dimensions of the identified fibers are shown in a separate window during testing and automatically recorded for possible use in epidemiological research. The automated counts afforded by the developed software showed good correlation with manual counts for the field samples with medium to high concentrations of fibers [49]. The counts were, however, less accurate at low fiber concentrations, possibly because of the interfering autofluorescent dust particles. To improve the accuracy of fiber identification and enable quality control, we also implemented a correction mode that could be used by professional asbestos analysts.

4.4 Development of a compact, robust and portable fluorescence microscope

Laboratory-grade fluorescence microscopes are not designed for operation under harsh environmental conditions generally observed at demolition sites and many other locations where there is a danger of asbestos fiber release. A more compact, light-weight and portable, battery-operated microscope is needed to enable on-site asbestos detection. Such a microscope should also be robust enough to tolerate outdoor use yet inexpensive to facilitate the adoption of the FM method. The combination of an advanced optical interface with computational power, data connectivity, large memory volume and low cost make smartphones and tablets an ideal platform for imaging [13]. Indeed, fluorescence microscopes have been developed with additive manufacturing and integrated onto the existing camera module of a smartphone [50–53]. Using a blue LED as a source of excitation light, we developed a microscope that integrates objective and eyepiece lenses, excitation/emission filters and dichroic

Table1. Potential peptide and protein aptamers against harmful nanomaterials

Materials	Plausible toxicity mechanism [a)]	Binding peptides or proteins
Fibers		
Chrysotile asbestos	Macrophage cell disruption	[27]
Amphibole asbestos	Macrophage cell disruption	[28, 45]
Carbon nanotube	Macrophage cell disruption	[65, 66]
Particles		
Ag	Generation of ROS and membrane disruption	[38, 67, 68]
Pt	ROS production	[69]
Al_2O_3	ROS production	[70]
SiO_2	ROS production	[26, 71–74]
TiO_2	Extensive disruption of alveolar septa	[38, 73, 75–78]
ZnO	ROS production, release of toxic cations with damage to cell membrane	[76, 79–82]
Fullerenes	ROS production, oxidant injury to cellular membranes	[21, 83]
Polystyrene	ROS production	[29]

[a)] Reported by Sharifi et al. (2012) [84]

mirror with an iPad camera (Fig. 4). With iPad connectivity, the sample images can be accessed from a remote asbestos laboratory in real time. The calculated spatial resolution of the portable fluorescence microscope was approximately 0.7 µm. During the initial tests, thin chrysotile fibers were clearly visible under the portable microscope. The performance of the portable microscope is sufficient for rapid on-site asbestos detection.

The FM method has been introduced in the "Asbestos Monitoring Manual" published by the Japan's Ministry of the Environment. The probes for the FM method are commercially available and have been purchased by several companies for asbestos monitoring. The formal validation of the FM method is currently underway in Japan. This effort involves a comparison with the existing methods, as well as cross-testing of the same samples by different labs to estimate the method's variability. The full validation data would be available for the future validation of the FM method by NIOSH.

5 Future applications of FM method to nanotoxicological studies

Nanotechnology is an emerging technology with a number of potential applications in the medical, optical, and electronic fields. However, several types of nanoparticles (particles <100 nm diameter) may adversely affect the environment and human health [54, 55]. Some researchers have predicted the emergence of a new type of pollution if nanoparticles are released into the environment [54, 55]. In animal experiments, exposure to multiwall carbon nanotubes has been shown to cause lung cancer and mesothelioma, showing the same effect as asbestos [56]. Nanoparticles generally have high surface redox reactivity, leading to potential toxicity effects for living cells that are not observed with larger particles [57]. An additional risk is the translocation of some nanoparticles from their site of deposition (usually the lungs) to the blood and even the brain [58]. Toxicologists have already begun to study the effects of nanoparticles on the brain and central nervous system [59, 60].

Given these risks, rigorous and effective regulation must be established for the manufacturing, safe handling and disposal of nanomaterials [61]. Over the past decade, guidelines for using nanomaterials at work have been developed by government agencies, academia, and occupational health organizations [61]. However, the development of measurement methods for nanomaterials remains a key research need because current detection methods generally rely on expensive and complex analytical instrumentation, such as TEM. To address these issues, some researchers have developed simple colorimetric detection assays based on the surface reactivity of nanoparticles [57].

Fluorescent labeling of material-binding peptides makes it possible to apply FM to the detection and measurement of nanomaterials. The key advantage of FM in the field of environmental biosensing is its ability to distinguish and selectively visualize target materials. Although the conventional FM methods cannot provide a spatial resolution below the diffraction limit (200–250 nm for visible light), the detection of fluorescent molecules below such limits is readily achieved. Indeed, the sensitivity of FM is sufficient for the detection of single chrysotile fibrils (30–35 nm in diameter) that are invisible under PCM (Fig. 3). Binding peptides for harmful or potentially harmful nanomaterials (carbon nanotube, silver, platinum, aluminum oxide, silica, titanium dioxide, zinc oxide, fullerenes, and polystyrene) have already been reported and are readily available for the simple counting of these particles/fibers (Table 1). However, most published research is not particularly concerned with specificity, focusing instead on the binding affinity to target materi-

als. It is therefore unknown how many of the identified material-binding peptides are highly specific to their targets and could be used to detect different inorganic materials. We have succeeded in visualizing titanium dioxide nanoparticles and carbon nanotubes using binding peptides (unpublished data). Although we still need to improve the specificity of the binding peptides, these results suggest that the FM method could be applied for the detection of environmental inorganic nanomaterials. It should also be noted that the resolution of the conventional FM is limited by optical diffraction, making it difficult to distinguish particles separated by less than several hundreds of nanometers. On the other hand, several super-resolution FM methods can already provide highly resolved optical images beyond the diffraction limit, allowing for the observation of even smaller nanoscale phenomena [62–64]. These methods will likely prove indispensable for nanotoxicological studies and should lead to novel applications of FM for the detection of nanomaterials.

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AK is an inventor on a patent (WO/2007/055243) issued to Hiroshima University that covers several asbestos-binding proteins. The patent does not alter the authors' ability to adhere to all Biotechnology Journal policies on sharing data and materials. AK and TN are board members of Siliconbio Inc. (Higashi-Hiroshima, Japan), which developed the portable microscope described in this review.

6 References

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Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Eiichi Tamiya and Chunhai Fan, covers advances in biosensors. This issue includes articles on optimization of biosensors for better sensitivity, exploration of the graphene interface for DNA analysis, aptamers for developing biosensors, etc. Image: © kentoh – Fotolia.com

Biotechnology Journal – list of articles published in the June 2016 issue.

Research Article

Quantification of total phosphorothioate in bacterial DNA by a bromoimane-based fluorescence method

Lu Xiao and Yu Xiang

http://dx.doi.org/10.1002/biot.201500432

Biotech Method

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Tatsuro Endo, Hiroshi Kajita, Yukio Kawaguchi, Terumasa Kosaka and Toshiyuki Himi

http://dx.doi.org/10.1002/biot.201500440

Biotech Method

Imaging of enzyme activity using bio-LSI system enables simultaneous immunosensing of different analytes in multiple specimens

Toshiki Hokuto, Tomoyuki Yasukawa, Ryota Kunikata, Atsushi Suda, Kumi Y. Inoue, Kosuke Ino, Tomokazu Matsue and Fumio Mizutani

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Rapid Communication

Aptamer-aptamer linkage based aptasensor for highly enhanced detection of small molecules

Van-Thuan Nguyen, Bang Hyun Lee, Sang Hoon Kim and Man Bock Gu

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