# Langmuir

# Repetitive Dissociation from Crocidolite Asbestos Acts as Persistent Signal for Epidermal Growth Factor Receptor

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Supporting Information

**ABSTRACT:** Mesothelioma is an incurable form of cancer located most commonly in the pleural lining of the lungs and is associated almost exclusively with the inhalation of asbestos. The binding of asbestos to epidermal growth factor receptor (EGFR), a transmembrane signal protein, has been proposed as a trigger for downstream signaling of kinases and expression of genes involved in cell proliferation and inhibition of apoptosis. Here, we investigate the molecular binding of EGFR to crocidolite (blue asbestos; Na<sub>2</sub>(Fe<sup>2+</sup>,Mg)<sub>3</sub>Fe<sub>2</sub><sup>3+</sup>Si<sub>8</sub>O<sub>22</sub>(OH)<sub>2</sub>) in buffer solution. Atomic force microscopy measurements revealed an attractive force of interaction (i.e., bond) as EGFR was pulled from contact with long fibers of crocidolite. The rupture force of this bond increased with loading rate. According to the Bell model, the off-rate of bond dissociation ( $k_{off}$ ) for EGFR was 22 s<sup>-1</sup>. Similar experiments with riebeckite crystals, the nonasbestiform variety of crocidolite, yielded a  $k_{off}$  of 8 s<sup>-1</sup>. These  $k_{off}$  values on crocidolite and riebeckite are very rapid



compared to published values for natural agonists of EGFR like transforming growth factor and epidermal growth factor. This suggests binding of EGFR to the surfaces of these minerals could elicit a response that is more potent than biological hormone or cytokine ligands. Signal transduction may cease for endogenous ligands due to endocytosis and subsequent degradation, and even riebeckite particles can be cleared from the lungs due to their short, equant habit. However, the fibrous habit of crocidolite leads to lifelong persistence in the lungs where aberrant, repetitious binding with EGFR may continually trigger the activation switch leading to chronic expression of genes involved in oncogenesis.

# INTRODUCTION

Mesothelioma is an incurable form of cancer located in the pleural lining of the lungs, peritoneal lining, and pericardium. This cancer is associated almost exclusively with long, narrow mineral fibers of asbestos. While this association has been known for decades,<sup>1</sup> the incidence of mesothelioma continues to grow and treatment options are still very limited. Over 20 million people in the United States are at risk of developing mesothelioma due to asbestos exposure,<sup>2</sup> and at least 500 000 deaths are predicted from mesothelioma in developed countries between 2005 and 2045.<sup>3</sup> Recent reports also document that humans, including children, may be exposed to significant levels of asbestos through the environment.<sup>4</sup>

Asbestos is a not a single mineral or material. Rather, it is a collective term referring to six naturally occurring silicate minerals (actinolite, anthophyllite, chrysotile, cummingtonite-grunerite, riebeckite, and tremolite) that exhibit an asbestiform habit (i.e., longitudinal parting into very thin fibrils). The asbestiform varieties of cummingtonite-grunerite and riebeckite are known commercially as amosite and crocidolite, respectively. These six asbestiform minerals do *not* all have the same potential for instigating disease upon exposure. Crocidolite

 $(Na_2(Fe^{2+},Mg)_3Fe_2^{3+}Si_8O_{22}(OH)_2)$ , also known as blue asbestos, is often regarded as the most carcinogenic type of asbestos.<sup>2,5,6</sup> Throughout this paper, crocidolite will be used to refer to the asbestiform variety of riebeckite.

Previous work has shown that aberrant activation of cell signaling cascades, like those associated with epidermal growth factor receptor (EGFR), may be a critical factor in asbestosassociated carcinogenic responses.<sup>6,7</sup> A study using immortalized human mesothelial cells revealed that long crocidolite fibers were physically associated with increased expression of EGFR on the surface of the cells.<sup>8</sup> Other studies with murine mesothelial cells have shown that crocidolite fibers cause phosphorylation of the intracellular domain of EGFR as well as upregulation of EGFR mRNA and protein biosynthesis.<sup>9,10</sup> Experiments with human endothelial cells have confirmed EGFR signaling and observed angiogenesis induced by direct contact with crocidolite asbestos.<sup>11</sup>

Received:February 11, 2013Revised:April 2, 2013Published:May 3, 2013

EGFR (also known as ErbB-1) is part of the ErbB family of proteins that play a critical role in initiation or perpetuation of signal transduction cascades that regulate cell development, proliferation, apoptosis, differentiation, and oncogenesis.<sup>12</sup> EGFR is a transmembrane, signal protein located on human mesothelial and lung epithelial cells. EGFR responds to external stimuli (e.g., hormones or cytokines) that bind to its extracellular ligand-binding domain (~620 amino acids). This initiates an extracellular signal that is transmitted via a membrane-spanning domain to a tyrosine kinase on the cytoplasm side of the plasma membrane.<sup>13</sup> Phosphorylation of the intracellular kinase domain (~550 amino acids) triggers a signal transduction cascade resulting in the expression of genes associated with increased cell survival or proliferation, and inhibition of apoptosis.<sup>13</sup>

In this report, we attempt to test whether crocidolite is capable of eliciting a biological response similar to that of the natural agonists of EGFR (e.g., epidermal growth factor, EGF, and transforming growth factor alpha,  $TGF\alpha$ ). Others have shown that the strongest biological response of EGFR is stimulated by those ligands that exhibit the highest dissociation rate constant  $(k_{\text{off}})^{14-16}$  Therefore, we determined the "biological response" that crocidolite may induce when it binds-unbinds to EGFR. Briefly, the  $k_{\rm off}$  value for EGFRcrocidolite was determined by using atomic force microscopy (AFM) in combination with the Bell model<sup>17,18</sup> to analyze the bond rupture force at different loading rates. Using this approach, we determined that the  $k_{off}$  value of EGFRcrocidolite is more rapid than published values for binding of EGFR to its natural ligands. This provides a fundamental, mechanistic explanation for the hypothesis that rapid association and dissociation of EGFR with crocidolite could repeatedly trigger cell signal cascades, particularly for durable crocidolite fibers that persist in the lungs indefinitely.

#### MATERIALS AND METHODS

**Samples and Reagents.** EGFR (ErbB1) from human carcinoma A431 cells (Sigma Aldrich, St. Louis, MO) was suspended in sterile phosphate buffered saline, PBS (~250  $\mu$ g mL<sup>-1</sup>), and stored at 4 °C for no more than a few hours until the time of experiments. Standard mineral samples of crocidolite (also known as blue asbestos) were acquired from the National Institute of Environmental Health Sciences in North Carolina.<sup>19</sup> The diameter of crocidolite fibrils and bundles of fibrils (fibers) ranged from 0.1 to 0.3  $\mu$ m, with varying lengths.<sup>19</sup> The riebeckite sample was purchased from a private collector (Papineau, Quebec). The riebeckite specimen lacked the fibrous habit of crocidolite and crystals were typically 10–100  $\mu$ m in length along the longest axis.

As shown in Figure 1, crocidolite and riebeckite are simply different growth habits of the same mineral  $(Na_2(Fe^{2+},Mg)_3Fe_2^{3+}Si_8O_{22}(OH)_2)$ . The crocidolite is ribbon-like and bounded primarily by {100} surfaces. The crystallites of riebeckite are characterized by well-developed {100} surfaces, but {110} faces are also present. These crystallographic forms were determined from extinction angle measurements, most of which were near 0°, a characteristic of a {100} surface in monoclinic amphiboles.

Mineral samples were used as is without being crushed but were washed in acetone followed by ultrapure water. Mineral specimens were fixed to a glass coverslip by gently heating on a warm hot plate. The fixed mineral samples were rinsed again with water before AFM experiments. Both riebeckite and crocidolite are insoluble in water,<sup>20,21</sup> and they are not expected to dissolve during the course of these experiments. It is important to note that the surfaces used in these experiments consisted of mineral growth surfaces as opposed to cleavage surfaces. Mineral growth surfaces represent those crystal planes that formed in nature as the result of igneous and metamorphic





**Figure 1.** Light microscopy images of crocidolite (A) and riebeckite (B). These are two different growth habits (asbestiform vs nonasbestiform) of the exact same amphibole mineral  $(Na_2(Fe^{2+},Mg)_3Fe_2^{3+}Si_8O_{22}(OH)_2)$ . Panel A shows the AFM cantilever positioned over a fiber of crocidolite. AFM measurements were performed on crystal growth faces of both samples as opposed to cleavage fragments (e.g., notice the bent crystal, as opposed to broken fragment, of riebeckite in top right area of panel B). Scale bar in both panels is ~100  $\mu$ m.

processes. We did not prepare samples by grinding crocidolite asbestos or riebeckite with a mortar and pestle.

**AFM Probes.** For the AFM experiments, we selected narrow, 200  $\mu$ m long, V-shaped silicon nitride cantilevers (Veeco Probes, Camarillo, CA) that had no visible defects at 400× magnification. The cantilevers were cleaned with acetone and ultrapure water, and dried with nitrogen gas. The tip-end of the probe was incubated for up to 3 h in a 30  $\mu$ L droplet of EGFR solution (~100  $\mu$ g mL<sup>-1</sup>) and then rinsed in PBS just before AFM experiments. The spring constant of

each probe was calibrated using a reference cantilever of known rigidity, yielding spring constant values between 0.04 and 0.10 N m<sup>-1</sup>.

**AFM of EGFR**–**Mineral Interactions.** EGFR-modified probes were used to collect force measurements on fibers of crocidolite (Figure 1A) or crystals of riebeckite (Figure 1B) in phosphate buffered saline (PBS) solution using a Nanoscope IV Bioscope AFM (Veeco-Digital Instruments). The deflection of the cantilever was recorded as the probe was repeatedly brought into contact and then retracted from the mineral. The force of binding (in pN) was determined as the product of the spring constant (pN nm<sup>-1</sup>) and the amount of bending (in nm) of the cantilever during retraction of the probe. The velocity of the retracting probe varied from 500 to 14 000 nm per second.

Several different slides (see above) were prepared with specimens of crocidolite or riebeckite. For each slide, AFM measurements were conducted on at least three different crystals of crocidolite or three different crystals of riebeckite. The AFM probe touched down and up at least 50 times at each of several different spots along each crystal (e.g., see Figure 1A). This yielded in excess of 10 000 curves for statistical analysis.

Raw data were converted to force versus separation plots (see comparison summary in Table S1 in Supporting Information) in a semiautomated fashion by using SPIP software (Image Metrology, Hørsholm, Denmark) and Microsoft Excel (Microsoft, Redmond, WA).<sup>22</sup> The magnitude of rupture (i.e., unbinding force, *F*) was taken as the final attractive binding event along the force-separation curve, according to refs 23–26 (see Figure S1 in Supporting Information). Velocity was converted to loading rate (*r*) for each force curve by multiplying the velocity of the probe by the slope of the force curve just before bond rupture (e.g., nm s<sup>-1</sup> × pN nm<sup>-1</sup> = pN s<sup>-1</sup>).<sup>25,27,28</sup>

Rupture forces were binned according to the loading rate (e.g., 0- 999 pN s<sup>-1</sup>, 1000–1999, 2000–3999, 4000–7999, etc.). The low and high extremes were grouped so that each bin contained a sufficient number of data points to compute an average. Because most of the distributions of the rupture forces per bin could be characterized by a Weibull distribution,<sup>29</sup> the midpoint loading rate and the scale (or 63.2 percentile) of the force distribution in each bin were also determined (see Figure S2). The mean rupture force (or scale parameter) and midpoint-loading rate of each bin were then used to construct a Bell plot<sup>17,18</sup> of the unbinding or rupture force (*F*) as a function of the loading rate (*r*) according to<sup>25</sup>

$$F = \frac{k_{\rm B}T}{x_{\beta}} \ln\!\left(\frac{rx_{\beta}}{k_{\rm off}k_{\rm B}T}\right) \tag{1}$$

where  $k_{\rm B}$  and T are the Boltzmann constant and temperature, respectively. According to this equation, the off-rate of bond dissociation ( $k_{\rm off}$ ) and the proportionality factor ( $x_{\beta}$ ; i.e., the lengthscale of bond dissociation) can be determined from a linear fit of Fversus ln r, where the slope equals  $k_{\rm B}T/x_{\beta}$  and the x-intercept (F = 0) is equal to  $k_{\rm off} = rx_{\beta}/k_{\rm B}T$  (see Figure S1). The loading rate (r; N s<sup>-1</sup>) was varied in these experiments by using cantilevers with different spring constants (N m<sup>-1</sup>) and changing the scanning velocity (m s<sup>-1</sup>).

Linear regression analysis with 95% confidence was computed by first transforming the loading rate to the natural logarithm, and then using the standard error of the fit of the force data to calculate 95% confidence intervals (e.g., fitted  $F \pm$  SD). All statistical analyses were performed with MiniTab v. Fifteen (MiniTab, State College, PA).

AFM Control Experiments. Control experiments were conducted by using EGFR-modified probes on silica glass  $(SiO_2)$  as well as uncoated AFM tips on the mineral specimens. In some cases, the EGFR-probes were reacted with a mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) targeted to the extracellular domain of the EGFR protein. The EGFR-modified tip was incubated with IgG2a (5  $\mu$ g mL<sup>-1</sup>) for 10 min and then rinsed before AFM experiments.

**Confocal Laser Scanning Microscopy (CLSM).** CLSM was used to obtain images of anti-EGFR adsorbed to crocidolite asbestos. Imaging was accomplished with a Meta510 confocal scanning laser microscope (Carl Zeiss Microimaging, Thornwood, NY) and an argon laser at an excitation wavelength of 488 nm at 5% emission. Crocidolite fibers were incubated for 2 h at room temperature with 10  $\mu$ g/mL of anti-EGFR IgG2a (in PBS) tagged with AlexaFluor 488 (sc-120, SantaCruzBiotechnology, Santa Cruz, California). This antibody is targeted to the external domain of the EGFR protein. Fibers were washed in triplicate and then treated with a droplet of Vectashield mounting medium (VectorLabs, Burlingame, CA) per manufacturer's instructions.

## RESULTS AND DISCUSSION

The first step in mesothelioma is physical contact between inhaled asbestos particles and receptors exposed on the outer surface of cells that line the lungs. The progression of mesothelioma may be dependent in part on the ability of crocidolite asbestos to induce or trigger transmembrane, signal-cascade proteins like EGFR<sup>6,8–11</sup> that regulate cell proliferation and apoptosis.<sup>13</sup> In this study, we try to determine if EGRF binding to crocidolite "ligand" elicits a response similar to natural cytokine agonists of EGFR (i.e., epidermal growth factor, EGF, and transforming growth factor alpha, TGF $\alpha$ ).

A series of published reports illustrate that for EGFR the physiological response is determined in large part by the dissociation rate constant ( $k_{off}$ ) of its ligands.<sup>14–16</sup> Lenferink et al.<sup>16</sup> nicely summarize this relationship as follows: "a more intense receptor signaling is induced by a ligand that in a dynamic manner is rapidly associating and dissociating, than by a ligand that is more or less irreversibly attached to its receptor". In other words, EGFR-ligands with the fastest  $k_{off}$  elicited the strongest biological response.<sup>15,16</sup> Therefore, we have used AFM to determine whether EGFR binding to crocidolite as a foreign "ligand" could elicit a  $k_{off}$  response similar to that of published values for natural ligands produced in humans.

Force Measurements for EGFR on Crocidolite Asbestos. AFM was used to directly probe the dissociation reaction that occurred when EGFR was pulled from contact with the longitudinal growth surface of crocidolite in PBS buffer solution (Figure 1A). The force (pN) necessary to rupture the EGFR– crocidolite bond was determined as a function of the loading rate (pN s<sup>-1</sup>). The off-rate of bond dissociation ( $k_{off}$ ) was determined by using the Bell model shown in eq 1. While this model is often applied to ligand–receptor bonds where both entities are organic molecules, many groups also use this approach for organic molecules decoupling from inorganic substrates like silicon nitride, gold, and mica.<sup>30–35</sup> Therefore, we used a similar approach for EGFR and the inorganic substrate crocidolite.

Figure 2A shows unbinding events that were observed when EGFR on an AFM probe was pulled from contact with crocidolite. In some instances, the protein broke free of the mineral at ~0 nm separation, and the cantilever returned to its resting position. Such binding events can be attributed to "nonspecific" adhesion.<sup>26,36,37</sup> In other instances, the EGFR molecules were extended before breaking free of the mineral surface (e.g., see Figure 2A). This type of "specific" binding resulted in a nonlinear profile (i.e., sawtooth)<sup>26,38-40</sup> as the bridging EGFR was unraveled or extended during the pulling process. The frequency of observing an unbinding event was 37% for EGFR-crocidolite (Table 1). Ideally, this frequency should be ~30% to ensure single molecule interactions.<sup>28,41</sup>

Control experiments were conducted to verify that the attractive interactions observed in Figure 2A were the result of the unbinding of EGFR from crocidolite. AFM measurements with uncoated AFM tips did not yield an attractive interaction



**Figure 2.** Individual retraction force profiles. Forces (in picoNewtons) are shown with respect to the separation (in nanometers) between an EGFR-modified probe and (A) crocidolite (i.e., asbestiform riebeckite) or (B) nonasbestiform riebeckite in saline buffer (PBS). The same AFM probe was used on both minerals in these plots.

Table 1. Frequency of Binding to  $EGFR^{a}$ 

|                 | crocidolite | riebeckite | glass |
|-----------------|-------------|------------|-------|
| % freq          | 37          | 18         | 18    |
| S               | 4           | 3          | 1     |
| n               | 68          | 27         | 8     |
| <i>p</i> -value |             | 0.001      | 0.001 |

"Values of mean percent frequency (% freq) reflect all AFM force measurements, which here are not grouped according to loading rate. The *p*-value is from the two-sample *t*-test to assess the null hypotheses % freq<sub>croc</sub> = % freq<sub>rieb</sub> and % freq<sub>croc</sub> = % freq<sub>glass</sub>. *S* is the variance, and *n* is the number of proportions.

with crocidolite (see Figure S3). Force measurements with an EGFR-tip on glass  $(SiO_2)$  exhibited significantly fewer binding events (Table 1). Those binding events that were observed for an EGFR tip on glass were mainly of the nonspecific type described above. Finally, a control experiment was conducted with an EGFR-modified probe incubated with an antibody that reacts with the extracellular domain of EGFR. Although a decrease in binding was expected, the anti-EGFR coated tip actually yielded a significant increase in binding frequency to crocidolite from 37% for an EGFR-coated tip to 57% for an anti-EGFR-coated tip.

Others typically observe diminished binding in similar control experiments when an antibody is injected into the AFM fluid cell.<sup>24,26,28</sup> However, there is one important difference in our experiment compared to those of others. We used an inorganic crystalline solid rather than a biological receptor. One explanation for the increased binding is that the EGFR antibody has an avidity for crocidolite. Indeed, this appears to be the case as confocal laser scanning microscopy

shows anti-EGFR coatings on asbestos fibers (see Figure 3). The significant difference in binding in the presence of anti-



**Figure 3.** Fluorescence imaging of anti-EGFR (yellow) and crocidolite (blue). The binding of the antibody to asbestos is seen here as a delineation of dye along the fibers. Two emission filters were used to separate the fluorescence of crocidolite from the dye. A bandpass range from 456 to 477 nm (A) shows the natural fluorescence of the crocidolite, whereas at wavelengths 531–606 nm (B), only the anti-EGFR AlexaFluor 488 conjugate was visible. Part C shows the composite fluorescence. The scale bar of 20  $\mu$ m applies to all panels in this figure. A control image (not shown) of a commercially prepared slide (Invitrogen, Carlsbad, California) with the same two filters confirmed that AlexaFluor 488 was not visible in the 456–477 nm range.

EGFR as well as results from the other control experiments noted above verify that the force spectra for the EGFR-coated tips (Figure 2A) are consistent with a particular binding signature for crocidolite.

Determining  $k_{\text{off}}$  and  $x_{\beta}$  for EGFR Dissociation from Asbestiform Crocidolite. Equation  $1^{17,18}$  was therefore used to determine  $k_{\text{off}}$  and  $x_{\beta}$  for EGFR and crocidolite. Figure 4 shows the rupture or unbinding force (*F*) as a function of loading rate (*r*). A positive correlation is observed between *F* and *r* for binding of EGFR to crocidolite fibers ( $R^2 = 0.95$ ; Figure 4B). Table 2 shows the values for  $k_{\text{off}}$  and  $x_{\beta}$  determined using the slope and intercept as defined in eq 1. The  $x_{\beta}$  value was determined to be 0.7 Å. The magnitude of this value is consistent with others who have used eq 1 to analyze binding reactions between ligand—receptor pairs<sup>42,43</sup> as well as



**Figure 4.** Rupture or unbinding force (*F*) plotted as a function of loading rate (*r*). Black dots correspond to EGFR unbinding from crocidolite, whereas open circles represent the EGFR-riebeckite bond. (A) Logarithmic scatterplot of *F* and *r* of all AFM force-separation curves. (B) Semilogarithmic plot of *F* as a function of *r* for the EGFR-crocidolite and EGFR-riebeckite bonds. The slope and *x*-intercept are used to determine  $k_{off}$  and  $x_{\beta}$  according to eq 1 (see values in Table 2).

Table 2. Summary of Dynamic Force Spectroscopy Values for EGFR-Mineral Binding $^a$ 

| mineral     | parameter | $x_{\beta}$ (nm) | $k_{\rm off}~({\rm s}^{-1})$ | $\tau$ (ms) | $R^2$ |
|-------------|-----------|------------------|------------------------------|-------------|-------|
| crocidolite | mean      | 0.07             | 22                           | 45          | 0.95  |
| crocidolite | 95% CI    | 0.05, 0.10       | 7,40                         | 25, 149     |       |
| riebeckite  | mean      | 0.05             | 8                            | 131         | 0.82  |
| riebeckite  | 95% CI    | 0.03, 0.15       | <1, 32                       | 31, 992 s   |       |

<sup>*a*</sup>Results are from linear regression of eq 1 for rupture *F* in response to the natural logarithm of *r* for EGFR on crocidolite or riebeckite. The bond lifetime  $(\tau)$  was determined as  $1/k_{\text{off}}$ .

instances where the interacting pair consists of an organic molecule and an inorganic solid.<sup>30,31</sup> The off-rate of bond dissociation  $(k_{\text{off}})$  value was determined to be 22 s<sup>-1</sup>, which corresponds to a bond lifetime  $(1/k_{\text{off}})$  of about 45 ms (Table 2). As shown in Table S2, analysis using a Weibull distribution<sup>29</sup> (Figure S2) yielded similar results for  $k_{\text{off}}$  (24 s<sup>-1</sup>) and  $x_{\beta}$  (0.6 Å).

Determining  $k_{off}$  and  $x_{\beta}$  for EGFR Dissociation from Nonasbestiform Riebeckite. To check these values of  $k_{off}$ and  $x_{\beta}$ , we also used an EGFR-tip to measure binding reactions on riebeckite. This specimen was selected because riebeckite has a different growth habit (i.e., nonfibrous) than crocidolite (Figure 1). That is, the riebeckite sample chosen and the NIEHS crocidolite are the same mineral but occur in two different forms. Therefore, one might expect similar reactivity because both samples of this mineral have the same bulk composition.

Figure 2B shows force spectra for EGFR pulled from the surface of riebeckite. The frequency of binding to riebeckite was significantly less than that of crocidolite (Table 1). Perhaps, this indicates a better "fit" or more consistent surface interaction for EGFR on crocidolite with its fibrous habit. Like crocidolite, there is also a positive correlation between the rupture and loading rate for EGFR on riebeckite ( $R^2 = 0.82$ ; Figure 4B). Therefore, eq 1 was used to determine  $k_{off}$  and  $x_{\beta}$  for EGFR-riebeckite of 8 s<sup>-1</sup> and 0.5 Å, respectively (Figure 4B; Table 2). While the calculated  $k_{off}$  value for crocidolite appears to be more rapid than that of riebeckite (Table 2), an ANOVA test to determine the significance of this apparent difference shown in Figure 4 yields a *p*-value >0.05.

Dynamic Force Spectroscopy on ErbB Family Proteins Similar to EGFR (ErbB-1). To the best of our knowledge, this is the first time that AFM dynamic force spectroscopy has been used to analyze ligand-EGFR interactions where the "ligand" is a mineral. No one has used AFM to probe EGFR receptors binding to its native ligands like EGF or TGF $\alpha$ . However, others have recently used the same AFM technique to measure  $k_{\text{off}}$  and  $x_{\beta}$  for receptors in the same ErbB family as EGFR (ErbB-1) as well as similar receptors for TGF. For example, Shi and co-workers collected AFM dynamic force spectra for the heregulin  $\beta$ 1 (ligand) and ErbB-3 receptors on living cells.<sup>44</sup> Additionally, Yu and colleagues collected AFM dynamic force spectra for TGF $\beta$ 1 (ligand) and its TGF receptors on living cells.<sup>26</sup> In these studies, specific ligand-receptor binding events were observed <30% of time, similar to our results (Table 1). Values for  $x_{\beta}$  in these studies ranged from 0.7 to 3.2 Å, and  $k_{\text{off}}$ ranged from 0.7 to 6.1 s<sup>-1</sup> (Table 3).<sup>26,44</sup> The similar magnitude of these values compared to our measurements on inorganic solids supports the validity of our approach to determine the biological response  $(k_{\text{off}})$  stimulated by minerals like crocidolite.

Table 3. Comparison of Off-Rate  $(k_{off})$  for EGFR (ErbB-1) Dissociating from Crocidolite (or Riebeckite) versus EGFR (or Similar Receptor Like ErbB3) in Complex with Natural, Biological Ligands (e.g., EGF, TGF, HRG)

| ligand–receptor pair <sup>a</sup> | $k_{\rm off}~({\rm s}^{-1})$ | method <sup>b</sup> | ref        |
|-----------------------------------|------------------------------|---------------------|------------|
| crocidolite-EGFR                  | 22                           | AFM                 | this study |
| riebeckite-EGFR                   | 8                            | AFM                 | this study |
| HRG-ErbB3                         | 2.4                          | AFM                 | 44         |
| TGF $\beta$ 1-TR $\beta$ II       | 0.2-6.1                      | AFM                 | 26         |
| TGFα-EGFR                         | 0.27-2.3                     | MBRL                | 14         |
| EGF-EGFR                          | 0.26-0.75                    | MBRL                | 14         |
| TGFα-EGFR                         | 0.27-2.0                     | MBRL                | 15         |
| EGF-EGFR                          | 0.16-0.66                    | MBRL                | 15         |
| superagonist EGF-EGFR             | 0.09-0.21                    | SPR                 | 16         |
| TGFα-EGFR                         | 0.04                         | SPR                 | 16         |
| EGF-EGFR                          | 0.03                         | SPR                 | 16         |
| EGF-EGFR                          | 0.03-0.07                    | SPR                 | 59         |
| EGF-EGFR                          | 0.002-0.01                   | FS                  | 45,60      |
|                                   |                              |                     |            |

<sup>*a*</sup>EGFR = epidermal growth factor receptor (also ErbB1); HRG = heregulin  $\beta$ 1, ligand; TGF = transforming growth factor, ligand; TR $\beta$ II = type II transforming growth factor receptor; EGF = epidermal growth factor, ligand. <sup>*b*</sup>AFM = atomic force microscopy; MBRL = mass balance of radioactive label; SPR = surface plasmon resonance; FS = fluorescence spectroscopy.

Comparing the Off-Rate ( $k_{off}$ ) of Natural Ligands to the Foreign "Ligand" Crocidolite. As noted above, the  $k_{off}$ value of cytokine or hormone ligands that bind to the extracellular domain of EGFR strongly correlates with the biological response *in vivo*. Two of the best studied ligands for EGFR are mitogenic signals: the 53-residue epidermal growth factor (EGF)<sup>45</sup> and 50-residue transforming growth factor alpha (TGF $\alpha$ ), which share 40% sequence identity.<sup>46</sup> Although the ligand-binding site on EGFR differs for EGF and TGF $\alpha$ ,<sup>47</sup> the biological effect induced by these two ligands is quantitatively similar in most cases. In some instances, TGF $\alpha$ functions as a more potent agonist of EGFR.<sup>16</sup>

The published  $k_{\rm off}$  values for EGFR binding to EGF range from 0.002 to 0.75 s<sup>-1</sup>, and those for TGF $\alpha$  range from 0.04 to 2.3 s<sup>-1</sup> (Table 3).<sup>14–16,46</sup> One series of  $k_{\rm off}$  values from Table 3 is particularly noteworthy. Lenferink et al.<sup>16</sup> determined the offrate of EGFR and its native ligands (i) EGF and (ii) TGF, as well as (iii) some elegantly engineered EGF/TGF chimeric molecules that induce significantly greater mitogenic cell stimulation than EGF or TGF alone.<sup>48</sup> The measured  $k_{\rm off}$ values were greatest for the superagonist molecules (i.e., the chimeric ligands) followed by EGFR-TGF and finally EGFR-EGF (see Table 3). This mirrors the biological response, i.e., stimulation of mitosis, triggered by each ligand.<sup>16,48</sup>

**Crocidolite Acts as Persistent Switch When It Binds to EGFR.** Our measurements of  $k_{off}$  for EGFR on crocidolite and riebeckite are more rapid than even the highest off-rate for EGFR-EGF or EGFR-TGF $\alpha$  (Table 3). The fast off-rate of the EGFR-mineral bond, up to 40 per second (Table 2), suggests that persistent binding to crocidolite acts as an incessant switch mechanism. Such a repetitious extracellular trigger could in turn cause an intracellular signal cascade leading to cell division and other biological functions associated with EGFR. While the growth signal is "turned off" for natural hormones or cytokines like EGF, which are internalized and degraded by cells through endocytosis,<sup>12</sup> the biodurability of crocidolite asbestos<sup>21</sup> would exacerbate this switch mechanism. Crocidolite "fibres are insoluble and not metabolized, they remain in contact with cells and thus serve as persistent sources of signals."<sup>11</sup>

Recent research by Mijailovich and co-workers even suggests that the inherent habit of fibers (i.e., long, narrow particles) in contact with a cell imparts mechanical stress on the cell's surface, which can trigger a reaction. Using a simple mathematical model, they demonstrate that long, rigid mineral particles (e.g., asbestos) in contact with a cell in cyclic motion (e.g., due to tidal breathing) can deform the cell thereby generating an external, tensile force on the cell's receptors.<sup>49</sup> Complementary experiments with A549 cells showed that asbestos fibers induce a significant cytokine response (IL-8) in cyclically stretched cells.<sup>49</sup>

There is still one unresolved issue with respect to our experiments using nonasbestiform riebeckite. The rapid  $k_{off}$  of EGFR with riebeckite (Table 2) suggests that this mineral should be associated with mesothelioma just like crocidolite. However, most studies have shown that the effect of riebeckite in tumor development is minimal,<sup>50–52</sup> despite the fact that its bulk chemistry is identical to crocidolite. This apparent contradiction can be addressed by considering the biopersistence of long, narrow asbestos fibers relative to wider, shorter particles.<sup>53</sup> More equant-shaped riebeckite crystals or cleavage fragments are cleared from the body through the mucociliary escalator.<sup>54,55</sup> This essentially removes the riebeckite trigger. By contrast, the long, narrow habit of crocidolite fibers and their

insolubility ensure that they persist in the body for long periods of time.<sup>21</sup> The higher off-rate for EGFR and crocidolite may therefore work in tandem with a long residence time in the body to instigate carcinogenesis.

#### CONCLUSION

This study shows that crocidolite asbestos binds to EGFR in such a way that it can stimulate a potent signal for signal transduction cascades associated with mesothelioma. Both crocidolite and riebeckite have heightened  $k_{\rm off}$  values toward EGFR compared to published values for natural agonists like EGF and TGF. This is important because the signal cascade response of EGFR is dependent upon the  $k_{\rm off}$  value of its extracellular ligand.<sup>14–16,46</sup> Further, our work provides a fundamental mechanism to explain the increased activity of EGFR observed in studies of cells and animals subjected to crocidolite asbestos.<sup>6,8–11</sup> Crocidolite fibers, whose insolubility, size, and shape allow them to persist in the lung, pleura, or peritoneum, will act as persistent triggers for transmembrane signaling proteins like EGFR over the long latency periods (20 years or more) associated with asbestos-induced cancers.

The rapid  $k_{\text{off}}$  of EGFR from nonasbestiform riebeckite would also likely activate the EGFR protein, but the different habit (i.e., smaller equant shape) of riebeckite allows the body to clear these particles, and therefore the effect of riebeckite in tumor development is minimal. This indicates that mineral surface reactivity is indeed a key factor in the development of mesothelioma, and also points once again to the importance of considering both physical (size and shape) as well as chemical parameters in asbestos toxicity.

Additional considerations are necessary before the conclusions of this paper are generalized to all instances of asbestos- or other mineral-related cancers. For example, only mineral growth surfaces were examined in this study, as all surfaces on an asbestos fiber are growth planes (i.e., crocidolite does not cleave), and the riebeckite particles were crystals. While growth surfaces are important in environmental exposure (e.g., inhalation of dust from soils containing asbestos), workrelated exposure to riebeckite may also be associated with cleavage or breakage fragments. These may have different reactivity than growth planes. Our experiments were conducted with extracted EGFR as opposed to EGFR in its native state within a cell membrane. Therefore, future work should be conducted on cells (e.g., human carcinoma A431 cells) that express EGFR in its natural physiology orientation with appropriate neighbor molecules in the plasma membrane. Finally, we investigated only one of the six asbestos minerals. There are a number of other naturally occurring asbestiform minerals and materials that should be studied with this approach. Chrysotile  $(Mg_3(Si_2O_5)(OH)_4)$ , for example, is among the most abundant forms of asbestos, but it is typically considered the least carcinogenic of the six types of asbestos. This could be due to the fact that chrysotile is not nearly as biopersistent as crocidolite.<sup>20,21,54,56</sup> Alternatively, chrysotile may have different surface reactivity<sup>57</sup> such that it cannot function as an active trigger (small  $k_{off}$  value) for EGFR. Of course, our method is applicable to other particles that pose respiratory risks, like quartz, glass, and wollastonite fibers,<sup>11</sup> or even materials like carbon nanotubes.58

### ASSOCIATED CONTENT

#### **Supporting Information**

Tables S1 and S2 and Figures S1, S2, and S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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#### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

#### Notes

The authors declare no competing financial interest.

# ACKNOWLEDGMENTS

We thank Dr. H. Catherine W. Skinner, Dr. John Olesik, and four anonymous reviewers for their constructive criticism. Michael Brown and Chris Yakymchuk kindly helped with the light microscope images of riebeckite. S.K.L. gratefully acknowledges financial support from the National Science Foundation (EAR0745808). S.K.L. dedicates this manuscript to Marjorie A. Hartsock Lower.

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