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Antibacterial Activity of Copper-doped Montmorillonite Nanocomposites Prepared by Alkaline Ion Exchange Method

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Abstract: *The antibacterial activity of copper-doped montmorillonite nanocomposites (Cu-MMT) has been investigated. Copper loaded clay nanocomposites have been synthesised by alkaline ion exchange processes in media containing copper sulfate at 550°C. Characterisation studies on the composites were done by X-ray diffractometry (XRD), scanning electron microscope (SEM) and absorption spectra analysis. SEM results indicated the diffusion of copper to the inner montmorillonite layer and opening of presenting cracks on its surface. The XRD analysis showed the presence of copper oxide (CuO) structure intercalated within the clay mineral layers. The antimicrobial effects of doped montmorillonite powders against pathogen bacterial strains Escherichia coli and Staphylococcus aureus were tested in broth media. Quantitative test in liquid media clearly showed that copper-doped samples had viable cells reduction ability for testing strains. The excellent antimicrobial activity of the composites was observed on S. aureus. In addition, the composite showed good stability in water and good promise for use in water treatment.*

Keywords: Antibacterial activity, Cu-MMT, alkaline ion exchange, nanocomposite

1. INTRODUCTION

Antimicrobial materials have the ability of inhibiting the growth or even killing certain types of microorganisms. The search for products with antimicrobial properties has gained particular importance in various operations, such as in raw materials for cosmetics and pharmaceuticals, hospital and veterinary products, food manufacture and animal feeding, among others. To attribute antibacterial properties to an inert material, it is necessary to treat it with metallic ions with bacteriostatic nature, such as silver,[1–4] copper[4–8] and zinc.[1,9,10] Among them, copper is one of the most promising because of its biocompatibility and high affinity for the pathogenic microorganisms.

Copper ions in the small quantities are essential for various metabolic processes in most of the living organisms, while the higher amounts are potentially toxic. There are three primary mechanisms for the disinfectant activity of copper ions. First, metal ions bind to proteins and deactivate them. Second, metal ions can interact with microbial membrane, which causes structural change and permeability. Finally, metal ions interact with microbial nucleic acids and thus prevent microbial replication.[5,11]

The research on heavy metal/clay mineral surface interactions is still of great interest because of the well-known toxicity of heavy metal ions and the need for removal of these metals from aqueous solutions.[12,13] On the other hand, the research on the bactericidal properties of heavy metal exchanged montmorillonite (MMT) has increased interest since antibiotic treatment is becoming obsolete, as the overuse of antibiotics has led to an increase of bacteria resistant to antibiotics.[14]

In recent years, clay mineral-based antibacterial complexes have been prepared by a series of processes between the clay minerals and antibacterial substances. Recently, Rivera-Garza et al. reported the elimination of the pathogenic microorganisms, *E. coli* and *Enterococcus faecalis* from water by silver-loaded clinoptilolite/heulandite mineral. They found that antibacterial activities of the exchanged samples were a function of exchange level against *Pseudomonas aeruginosa* and *E. coli*.[15] In another work, a vermiculite–copper hybrid material shows strong antibacterial action against *S. aureus*.[16]

Guo et al. studied the adsorption properties of Cu^{2+} loaded montmorillonite (Cu-MMT) clays for *E. coli* K_{88}. Their methods for preparation of this composite were mixing and ion exchange of MMT and Cu^{2+} ($CuSO_4.^5H_2O$) in solution.[6] Bagchi et al. synthesised copper nanoparticle based clay composite by in-situ reduction of a copper ammonium complexion. They evaluated the antimicrobial activity of the composite against *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecalis*.[7] M. F. Santos evaluates the possibility of incorporating antibacterial properties in bentonite minerals by applying the ion exchange process.[3] These metals/clay composites usually prepared by ion exchange of positively charged metal ions and alkali ions present in a surface layer of clay or by synthesis of nanoparticles and immobilisation them on the clay by various techniques such as ion exchange processes and sol-gel.[1,5,6,8,9,16] Nevertheless, many of these techniques typically entail multiple steps combined with ultrasonication and stirring that take long hours or even days followed by chemical reduction of the precursor metal salts.

Despite the fact that the MMT surface is poorly covered by usual mentioned process,[4] the diffused copper to MMT structured via alkaline ion exchange are strong enough to form very stable and durable copper/ MMT nanocomposites. Furthermore, this method is fast and simple. In addition, preparation of Cu-MMT by this method only requires an oven and a salt containing the doping ions. Metal-alkali ion exchange in glass has been widely used to dope silicate glasses. The procedure is done by immersing the substrate in a molten salt bath containing the dopant ions, which replace alkali ions of the substrate matrix. Ion exchange is carried out by replacing monovalent alkali ions present in a surface layer of a glass substrate with different ions from a molten salt bath.

To the authors' knowledge, there have been no studies dedicated to antibacterial by alkaline ion exchange with Copper. The aim of this paper is to characterise and evaluate the antibacterial properties of MMT-Cu nanocomposite prepared via alkaline ion exchange method.

2. EXPERIMENTAL

2.1 Materials

Bentonite clay (Na-montmorillonite) used as the solid support for copper was obtained from Kanisaz Jam Company (Rasht, Iran). All reagents were of analytical grade and were used as received without further refinement. All aqueous solutions were prepared using distilled water. Muellerhinton agar broth and nutrient agar were purchased from Merck. The bacterial strains used for the antibacterial activity were Gram-negative *E. coli* (PTCC 1270) and Gram-positive *S. aureus* (PTCC1112) which were obtained from the Iranian Research Organization for Science and Technology.

2.2 Alkaline Ion Exchange with Copper

Bentonite were submitted to an ion exchange process by immersion to melts of $CuSO_4.5H_2O$ at 550°C–560°C for 10, 20, 40, 60 and 90 min. This operation was done using 5 g of bentonite and 5 g of $CuSO_4.5H_2O$. After the ion exchange, the bentonite was adequately washed with distilled water and sanitation. The principal aim of this step was to dissolve any compound that was not contained in the montmorillonite. After dissolution, the bentonite was dried in an oven.

2.3 Characterisation

Synthesised samples were analysed by X-ray diffractometry (XRD) (Philips PW 1050). The patterns were registered in the 2θ range from 10^o to 60^o with a scanning step size of 0.05º. MMT clay was used as the reference. The morphology of the molecules was investigated by scanning electron microscope (LEO 1430VP, Germany). Absorption spectra of MMT and Cu-MMT nanocomposites was measured by a UV–visible diffusive reflectance spectrophotometer (Sinco S4100, Korea), in the wavelength range 200–1000 nm.

2.4 Antibacterial Activity

The antibacterial activity of the samples was studied by plate count technique on *E. coli* and *S. aureus*. Typically, 50 mg of powder samples (MMT and Cu-MMT nanocomposites) was added to cultures of bacteria in 5 ml sterile Muellerhinton broth. The cultures were then incubated at 37ºC on a rotary shaker for 24 h. Growth inhibition with time was followed by plating 100 µl of the treated cultures on nutrient agar plates. Bacterial colonies were counted and compared with control after 24 h incubation at 37°C. The whole experiment was repeated twice to reproduce the data. The antibacterial effect was calculated using the:

$$\text{Mortality (\%)} = \frac{B - C}{B} \times 100$$

where B is the mean number of bacteria in the control samples (CFU/sample) and C is the mean number of bacteria in the treated samples (CFU/sample).

2.5 Leaching Test

In order to evaluate the stability of the nanocomposites, leaching tests were performed. For each composite material, 0.2 g was immersed in 10 ml of distilled water and vigorously shaken in a water-bath shaker (30°C, 200 rpm) for 3 and 6 h. Supernatant from each test tube was collected after 3 and 6 h by centrifugation at 10000 rpm for 10 min. Copper ion release from the Cu-MMT nanocomposites was qualitatively determined by using the atomic absorption spectroscopy analysis.

3. RESULTS AND DISCUSSION

Appearance and colour of the parent bentonite were white (Figure 1). Alkaline ion exchange of the bentonite by copper changed the colour of bentonite from grey for 5 min treated sample to cream for treating sample for longer times. It appears that this variation of colour goes up from the amount of loaded or ion exchanged copper onto the bentonite.

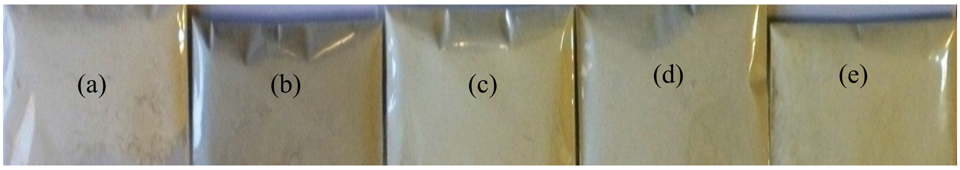


Figure 1: Photography of the parent bentonite (a), and ion exchanged bentonite with copper sulfate for 10 min (b), 20 min (c), 40 min (d) and 60 min (e).

3.1 Diffusive Reflectance Spectra

The formation of copper nanoparticles in the bentonite mineral system was first observed by UV-visible absorption spectra studies (Figure 2). The absorbance band in a UV visible spectrum of $CuSO_4$ shows two bands around 247 and 310 nm. These bands are attributed to a charge transfer transition of inter layer tetrahedral and octahedral atoms.[19] After alkaline ion exchange, the band of 247 nm disappeared and it has not been observed in Cu-MMT nanocomposites anymore. Absorption spectroscopy of bentonite revealed a characteristic band around 310 nm. Ion exchange of Cu^{2+} loaded bentonite results in formation of Cu-MMT nanocomposites. Evidence for this comes from the change in composite colour (Figure 1). This band red shifted between 3 and 12 nm (Figure 3) depending upon the particle size and shape. This means that increasing time of ion exchanged caused more diffused copper ion and coalescence of forming particles to each other.

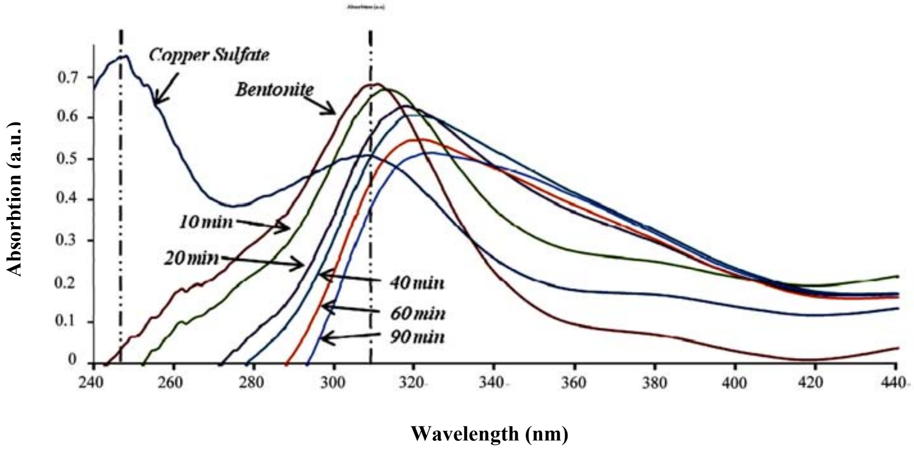


Figure 2: Diffusive reflectance spectra of prepared samples.

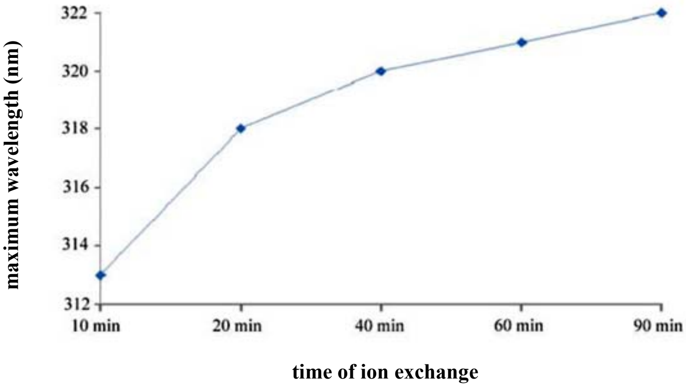


Figure 3: The wavelength of maximum absorbance of prepared samples.

Figure 4 shows the differences between maximum absorbance and the right shoulder (10 nm after the maximum wavelength of peak). As we can see from this figure, the absorbance of the right shoulder of each composite increased, thus the difference decreased. This means that the peak broadened by increasing ion exchange time. The broadness of the absorption band probably arises from the wide size distribution of copper nanoparticles. As the ion exchange time increased, the absorbance band slowly broadened in the copper-doped bentonite nanocomposites. Therefore, for an ion exchange time of less than 40 min, the change in distribution of copper nanoparticles is large, but beyond this time, the distribution becomes small.

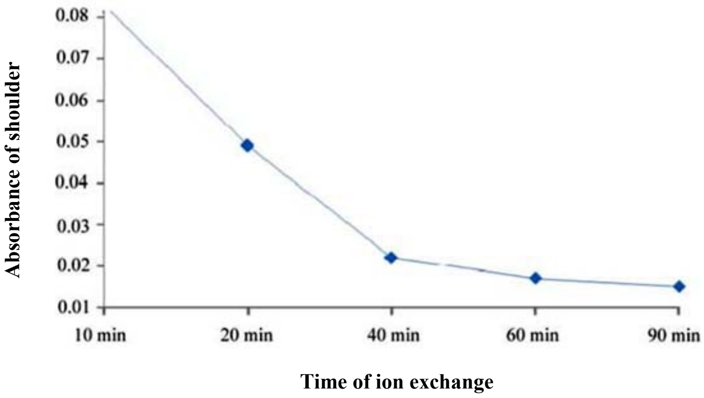
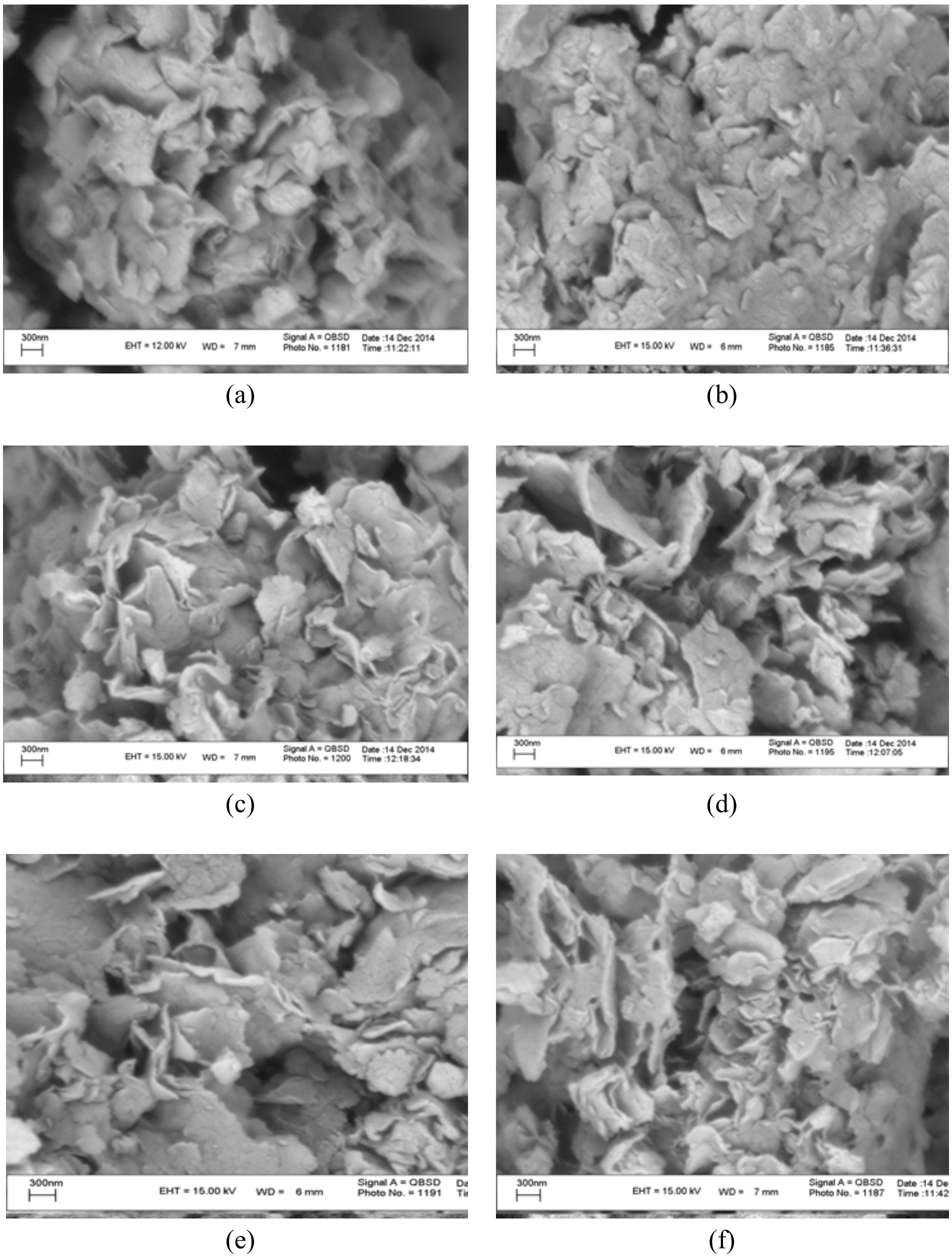


Figure 4: The absorbance of right shoulder of prepared samples.

3.2 SEM Analysis

The scanning electron micrograph (SEM) of MMT in Figure 5(a) shows the typical layered structure with numerous flakes of clay particles with a sheet-like morphology. In Cu-MMT, the clay structure is maintained with some changes. As can be seen from Figure 5, after alkaline ion exchange of MMT for 10 min, the porosity of clay obviously decreased. This is because of the filling of the pores of MMT by copper molecules. In this sample, copper still did not have enough time for diffusion to the inner MMT structure and only situated on the surface of MMT. Increasing the ion exchange time to 20 min caused the composite to return to the flaks-like structure, whereas the shape of the sample of 20 min is approximately the same with parent MMT. More flaks and more porosity can be seen in longer ion exchanged samples. This is because of the diffusion of copper to the inner layer of MMT and opening of crack on its surface. Thus, more layers can be seen on the composite surface.

In previous works, the treated clay contains heterogeneous particles on its surface.[8,13,16] On the other hand, metallic nanoparticles typically cover surfaces of alumino-silicate minerals. The authors' results of these studies obviously differ in compression. As can be seen from Figure 5, no particles are formed on the MMT surface after alkaline ion exchange. However, the structure of MMT has been changed and more flaks appeared. As a result, more porosity can be seen in the ion exchanged samples.



(a) (b)

(c) (d)

(e) (f)

Figure 5: SEM image of the parent MMT (a), ion exchanged for 10 min (b), 20 min (c), 40 min (d), 60 min (e) and 90 min (f).

3.3 XRD Analysis

The XRD patterns collected are presented in Figure 6. The patterns were smoothed by using an eight-point average, in an attempt to minimise the appearance of background noise. The reflections on 2θ values of about 35.17° (111) and 61.89° (113) correspond to copper oxide (CuO). The appearance of the oxide reflection in the diffractogram suggested the presence of CuO structure intercalated within the clay mineral layers. The intensity of the reflection corresponding to the CuO increases with the increase in the loading within the layered silicate. As shown by the sharp and more intense band, the degree of crystallinity increases with copper concentrations.

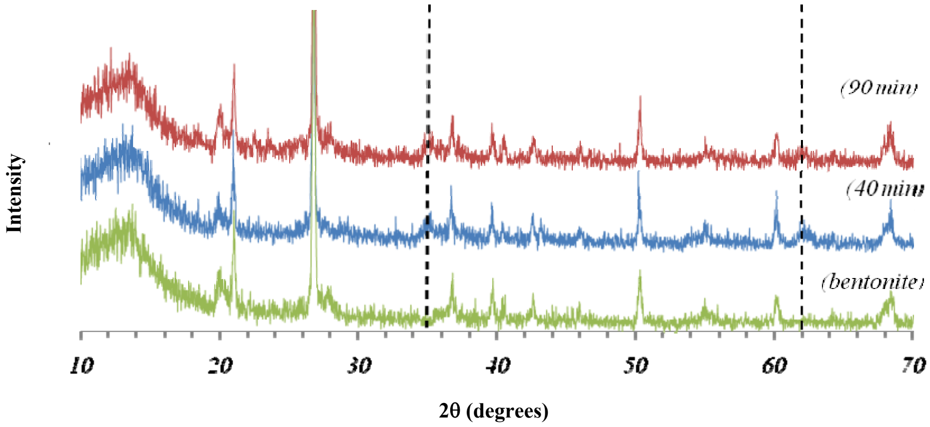


Figure 6: X-ray diffraction pattern of parent bentonite and Cu-MMT nanocomposites.

3.4 Antibacterial Assay

The influence of the time of ion exchange on the antibacterial activity of the sutures was studied against Gram-negative *E. coli* and Gram-positive *S. aureus*. The effects of different ion exchange time on the antibacterial properties of the Cu-MMT nanocomposites were presented in Table 1. The antibacterial trial showed that MMT had no antibacterial activity. The antibacterial activity of Cu-MMT tested for *E. coli* and *S. aureus* showed positive results with high mortality. The tests exhibited more than 99.98% mortality against *E. coli*, whereas it showed 100% mortality against *S. aureus*.

Table 1: Mortality percentage of Cu-MMT against *E. coli* and *S. aureus.*

Ion exchange time (min)	Mortality (%)	
	E. coli	*S. aureus*
10	99.96	85.23
20	99.97	95.35
40	99.98	100
60	99.98	100
90	99.99	100

The antibacterial property of copper nanoparticle is well researched and the mechanism of its action is generally found to be a combination of several types of interactions like membrane perforation, DNA damage, protein denaturation, etc. However, they do not equally affect all microorganisms. As studied earlier, bacterial susceptibility to nanoparticles depends on several factors like bacterial strain, nanoparticle type and size, the nature of growth media and initial cell concentration. In general, Gram-negative organisms are more resistant to the growth inhibiting effect of copper ions/nanoparticles than Gram-positive ones.[7]

Copper-based antimicrobial materials could easily compete with comparable silver-based materials, and would be much cheaper.[16]

3.5 Copper Ion Release Assay

Table 2 shows the copper amount released into the aqueous media as a function of the contact time between phases. Maximum release of copper ion was observed at around 6 h for 90 min sample with a corresponding increase in mortality rate for bacterial cell. The copper ion released from the composite may come from the nanoparticles either adsorbed on clay surface or suspended in the solution after desorption. The obtained results indicate, that the materials do not pose any danger for drinking water treatment since the leached metals were very small and in acceptable concentrations.

Table 2: Copper concentration in water at different contact times between the Cu-MMT and the aqueous media.

Ion exchange time (min)		Copper concentration in water (ppm)			
		MML	40	60	90
Contact time (h)	3	0	0.02	0.02	0.21
	6	0	0.07	0.05	0.38

The copper ion released from the alkaline ion exchanged Cu-MMT is obviously lower than previous studies.[8,15] This can be attributed to diffusion of copper to the MMT structure instead of attachment of copper nanoparticles to MMT. The advantage of this is that the antibacterial property of Cu-MMT composite would be longer.

The released Cu^{2+} would act directly on the bacteria adsorbed on the surface of the Cu-MMT, instead of into the medium and indirectly on the bacteria. In other words, the active Cu^{2+} density on mineral surface was much higher than its concentration in the solution. In summary, two possible mechanisms for the antibacterial activity of Cu-MMT were proposed. One involves the adsorption of the bacteria from solution and immobilised on the surface of the Cu-MMT. Alternatively, Cu^{2+} could disassociate from the clay surface and directly exert its antibacterial effect on the bacteria.[8]

4. CONCLUSION

The aim of this study was to evolutionise the antibacterial activity of copper-doped montmorillonite nanocomposites synthesised by alkaline ion exchange processes in media containing molten copper sulfate at 550°C temperature. The SEM results indicated the diffusion of copper to the inner layer of montmorillonite and opening of presenting cracked on its surface. The XRD analysis showed that the copper ions fully incorporated into the montmorillonite. Quantitative test in liquid media clearly showed that copper-doped samples had viable cell reduction ability for testing strains. The excellent antimicrobial activity of the composites was observed on *S. aureus*. In addition, the composite showed good stability in water. In the present form the clay composite shows good promise for use in water treatment.

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