

# Microwave assisted ultrafast immobilization of CuO nanostructures in paper matrices for antimicrobial applications

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## Research Article

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# Abstract

CuO nanoflakes with dimension of 50-150 nm × 200 nm were successfully immobilized on the surface of cellulose fibers by an ultrafast (5 min reaction time), scalable microwave assisted method. The achievement of high retention of CuO upto 70% has been phenomenal as the immobilization was carried out in the absence of any linker, binder or retention aid. The successful recyclability of the CuO immobilized paper matrices upto five consecutive cycles while retaining 87% of immobilized CuO nanostructures in paper matrices has been unprecedented. The antimicrobial activities of the paper matrices have been investigated by deactivating both *G. trabeum* and *E. coli*. The CuO immobilized paper matrices have desisted the growth of *G. trabeum* for upto 28 days, which is phenomenal and completely deactivated *E. coli* of the CFU in order of  $10^7$  in the presence of visible light with exposure time of 2 h only.

# Introduction

Paper, one of the first manmade engineered materials, have travelled through various ups and downs in its journey through the history (Matsubara et al. 1995). The last several decades of research in paper matrices were mostly devoted to the development of paper matrices for high-end applications owing to the demand in different avenues and cost reductions due to some restrictions imposed with the environmental concerns that increases the cost of raw materials (Matsubara et al. 1995; Gimenez et al. 2011; Li et al. 2017; Ornatska et al. 2011; Mostafa et al. 2019; Alammouz et al. 2019). As a material, the paper matrices stay firm among many advanced materials due to its biodegradability nature (Moon et al. 2011). The raw material, cellulose, is a biopolymer with a maximum natural abundance (Moon et al. 2011). In early 1960s, several paper industries started using metal oxides, clays and other ceramics to pertain certain properties in the paper matrices (Chauhan et al. 2012; Patel 2007). This journey continued in the same fashion for the next few decades until the evolution of the nanotechnology, which renewed the interest among the researchers to find new ways to incorporate these metal oxides and other fillers into the paper matrices with a controlled microstructure so that new and renewed properties and applications can be realized (Ogihara et al. 2012; Monk et al. 2001; Manekkathodi et al. 2010; Zeng et al. 2015; Fujiwara et al. 2017; Adel et al. 2016).

For the incorporation of the various metal oxides, hydroxides etc. nanostructures have been carried out by different approaches adopted by various research groups (Mostafa et al. 2020; Dong et al. 2018; Citak et al. 2019). Among these, the wet chemical approaches using various linker, binder and retention aid have dominated the research (Iguchi et al. 2003; Khatri et al. 2014). The added cost owing to the addition of these molecules and materials along with their negative impact on the application of the obtained paper matrices have further encouraged the researchers to find alternative approaches where none of these extra molecules and materials are used (Chauhan et al. 2015a). In this direction, our research group invented a hydrothermal approach, where neither of these linker, binder and retention aid was used to immobilize various metal oxide nanostructures such as ZnO, TiO<sub>2</sub>, Ce<sub>2</sub>O<sub>3</sub>, Fe<sub>2</sub>O<sub>3</sub> etc. on the surface of cellulose fibers of the paper matrices (Aggrawal et al. 2019; Aggrawal et al. 2015; Chauhan et al. 2015b).

One of the drawbacks of the hydrothermal approach is the long experimental time, which varied between few hours to a couple of days. However, the high retention of the nanostructures and versatile nature of the method has encouraged us further to find approaches where fast immobilization can be possible.

In the recent times, there is an utmost need for the development of safe and reliable antimicrobial systems. Pathogenic microbes are increasing day-by-day and the commonly followed antibiotics treatment is inactive against the recently emerged superbugs (antibiotic resistant bacterial species) (Wright 2000; Willyard 2017; Spellberg et al, 2008). Almost 80% of the bacterial infections are caused by Gram negative bacterial species (Mahmoodi et al. 2018). Another major problem lies in the water borne diseases which is due to the unavailability of safe drinking water to the major population of the world. Nano sized CuO has gained a lot of attention owing to its good chemical and physical stability (Mahmoodi et al. 2018). It has been applied in various applications such as sensing, semiconductor devices, supercapacitor, photocatalysis, catalysis and antimicrobial. It is a p-type semiconductor with a narrow band-gap of 1.3-1.8 eV (Maheswari et al. 2018). Furthermore, nanosized CuO has proven to be an excellent antifungal material. The CuO has been used as an antimicrobial agent because of its bio-compatibility, non-toxicity against mammals, easy and economical preparation (Maheswari et al. 2018). The nanomaterials need to be in contact with bacterial or fungal cells in order to provide antimicrobial properties. But nanoparticles tend to aggregate easily hence decrease the active surfaces. To achieve great applicability, they are incorporated or immobilized on an easily available and economical substrate. Further, the separation of the nanomaterials after the application is very difficult which can be easily sorted out using immobilization of active nanomaterials on some substrates.

Based on the above, in this research an ultrafast immobilization of CuO nanoflakes has been carried out using a microwave assisted approach, where the successful immobilization of the CuO nanostructures can be completed with an experimental time of only 5 min. Not only the fast immobilization of the nanostructures, but also the recyclability of the paper matrices for multiple preparation steps and antimicrobial activity against *E. coli* and *G. trabeum* in the presence of visible light have been investigated.

## Experimental

### Materials

Softwood pulp (Star paper mill, India), cupric sulphate (Chemlabs), sodium hydroxide (Himedia) and ethanol (Changshu Hongsheng Fine Chemical), were procured and used as such without any purification. The *E. coli* (MTCC no. 1698) and *G. trabeum* (MTCC No. 3168) were procured from Microbial Type Culture Collection and Gene Bank, Chandigarh, India.

### Synthesis

Copper oxide (CuO) nanoflakes in varied concentrations (5-30%) have been immobilized on the cellulose fibers of the paper matrices using single step, facile, ultrafast (5 min only), microwave assisted synthesis

method (Fig. 1). Typically, 1.6 g of softwood pulp was dispersed in 40 mL of deionized water. To it, 50 mL of xM of cupric sulphate solution already prepared in DI water was added and stirred at RT for 15 min, followed by dropwise addition of 40 mL of yM NaOH solution under continuous stirring at RT for 15 min. Composition of the precursors were added according to the wt. % of CuO nanoflakes immobilization and summarized in Table S2.

The complete reaction mixture has been transferred to the 250 mL round bottom flask and employed to microwave treatment in an open vessel system for 5 min at 50 °C and 300 W power. The fibers have been washed repeatedly with ethanol and DI water. It was then dried at RT followed by Handsheet preparation using British sheet former following TAPPI test method 205 sp-02. Several other CuO precursors have also been tested for immobilization of CuO nanostructures in the paper matrices (ESI for detailed synthesis).

## Characterization

Standard combustion test and thermogravimetric analysis (TGA) have been used to check the retention of immobilized CuO nanostructures in the paper matrices. In the combustion test, known weight of the paper matrices have been burnt in the muffle furnace at 520 °C for 5 h. It leads to complete oxidation of the organic matter i.e., cellulose leaving behind only inorganic copper oxide. For comparison, blank paper matrices (without CuO content) was also burnt under identical condition. The TGA analysis was carried out by heating the specimens in EXSTAR TG/DTA6300 with a heating rate of 5 °C per min in an argon atmosphere. The thermograms of all the specimens including the blank paper matrices have been compared and content of metal oxides have been estimated. The microstructure of the blank paper and CuO immobilized paper have been done using FESEM. In the present study, FESEM have been carried out using Gemini 500 FESEM (Zeiss) instrument. Standard gold sputtering has been carried prior to FESEM analysis. The crystal structure analysis of the cellulose fibers and CuO immobilized cellulose fibers have been carried out using XRD. In the present study, XRD patterns have been recorded on Rigaku Ultima IV with CuK $\alpha$  radiation ( $\lambda = 1.5405 \text{ \AA}$ ) at a scanning speed of 4°/min in the range of 10 to 70° of the 2 $\theta$  scale. The obtained diffraction patterns were compared with ICDD (International Centre for Diffraction Data) files using Xpert high score. The oxidation state and chemical environment of the different elements present in the specimen have been carried out using XPS. It was done on PHI-5000 VersaProbe III, ULVAC-PHI INC. The peaks were referenced at 284.8 eV, the standard binding energy of C1s. Monochromatic AlK $\alpha$  have been used as the X-ray source in the instrument.

## Antimicrobial activity

The paper matrices were subjected to the antimicrobial activity, i.e., antibacterial and antifungal test. The antibacterial activity tests have been carried out using blank paper and CuO immobilized paper matrices on *E. coli*, a Gram negative bacterium in the presence of visible light for 2 h. Experiments were carried out in accordance with standard antibacterial protocol ISO 20743 with minor modifications. The experiments were repeated in triplicate to remove any ambiguity and the results have been interpreted in percent reduction and log reduction values in *E. coli*. Typically, specimens were cut in the size of 8 cm  $\times$  1 cm and

were steam sterilized in an autoclave. These were then put into the 10 mL of *E. coli* suspension and incubated at 37 °C for 2 h in the presence of visible light in a bacterial incubator. After 2 h, serial dilutions of the *E. coli* suspension were prepared and plated on the LB plates which were incubated for 24 h in an incubator at 37 °C in inverted position. The *E. coli* colonies were counted and CFU was calculated. Identical experiments were repeated 3 times in order to estimate the standard deviations in log reduction. The antifungal activity has been performed against *G. trabeum*, a cellulose eating fungus which produce cellulase enzyme to destroy the paper matrices. Typically, already grown fungal colonies were picked up using bacterial loop and transferred to sterilized potato dextrose agar plates. Paper specimens were cut in the size of 2 cm × 2 cm and steam sterilized in an autoclave at 15 psig pressure for 20 min. These were then placed on the fungal colonies in the petriplates and incubated at 28 °C for upto 27 days. Photographs were taken at regular intervals to check the destruction of the paper matrices by *G. trabeum* and investigate the antifungal activity of the prepared CuO immobilized paper matrices.

## Results And Discussion

The retention of CuO nanoflakes in the paper matrices have been estimated using combustion test and TGA analysis carried out in argon atmosphere (Aggrawal et al. 2019; Aggrawal et al. 2015). The paper matrices burnt in air at 520 °C for 4 h in a muffle furnace could burn out the organic mass completely leaving behind the inorganic CuO nanostructures. The extent of immobilization can be easily investigated when the leftover mass after burning of the blank paper is compared with that of the immobilized paper matrices. The leftover mass of different paper matrices has been summarized in Table S3. To have an ambiguity, the retention of CuO in paper matrices have also been estimated using TGA experiments. While carrying out TGA in argon atmosphere (Fig. 2), it has been observed that around 12 % is remaining even in the blank paper after burning at 800 °C, this is due to the carbonization of the organic mass as the TGA has been carried out in the argon atmosphere. To calculate the retention of CuO using TGA, the remains of blank paper has been deducted from the remains of CuO paper and summarized in Table S3. The results of combustion test corroborate with the results of TGA analysis with minor deviations which may be due to the fact that combustion test is carried out in air whereas TGA is carried out in argon atmosphere. DTG curves are given in Fig. S1

As the immobilization was realized with ultrafast experimental time of 5 min only, there was a concern on unshackle of the immobilized nanostructures from the paper matrices. In order to address this concern as well as to test the efficacy of the recyclability of the paper matrices for multiple paper making cycles, the CuO immobilized paper matrices have been redispersed in water and followed the same steps of paper making for five consecutive cycles. It was phenomenal to see there was hardly any loss of the nanostructures during this recycling processes. A maximum of only 13 % loss of the nanostructures was estimated even after five cycles as shown in Fig. 3. The worth of immobilization can be realized by the reported fact that by conventional filler loading, paper lose almost 100 % fillers just after three cycles whereas in case of in-situ filler loading paper matrices retain only 30 % of fillers after six consecutive cycles (Ciabanu et al. 2009).

The microstructure of the CuO immobilized has been studied by FESEM as shown in Fig. 4. The clean surface of the cellulose fibers has been observed in blank paper matrices as shown in Fig. 4a and b. The uniform distribution of CuO nanoflakes have been observed in the high magnification images of specimen 5CuOP-30CuOP (Fig. 4b-f). The increase in content of CuO is clearly visible even from the FESEM images. In the specimen, 30CuOP, the surface of the cellulose fibers are almost covered with the CuO nanoflakes. With increase in content of CuO in the paper matrices, it has been observed that, clear nanoflakes have been immobilized on the paper matrices. The breadth of the nanoflakes vary in the range from 50-150 nm whereas length extend upto 200 nm.

The phase analysis of the paper matrices has been carried out using XRD (Fig. 5). Three broad peaks centered at d-values of 0.5823 and 0.3896 nm can be seen in all the specimens, which are attributed to the (101) and (002) reflections of cellulose-I, respectively (Aggrawal et al. 2019; Aggrawal et al. 2015). In the CuO immobilized paper matrices, additional peaks at d values of 0.4685, 0.3423, 0.5132 nm and 99.52, respectively have been observed which are assigned to the (-111), (200) and (-202) planes of the monoclinic phase of CuO (JCPDS file no. 041-0254) (Xu et al. 1999; Reddy 2017). These peaks are not clearly seen in the specimen 5CuOP, due to the fact that content of CuO is less and the small peaks is any are obscured in the diffraction pattern of the cellulose.

The oxidation state and chemical environment of every element present in the specimen has been confirmed using XPS analysis as shown in Fig. 6. The full survey scan of all the specimen is shown in Fig. 6a. In all the specimens, peaks for carbon and oxygen are present at 286.6 and 532.9 eV, respectively, coming from the cellulose (Aggrawal et al. 2019; Aggrawal et al. 2015). In the survey scan of CuO immobilized specimen, several peaks have been observed in the region of 930-970 eV which are originated from Cu and satellite peaks confirming the presence of Cu in the specimens (Cao et al. 2019). To confirm the chemical environment and oxidation state, high resolution C1s, O1s and Cu2p have been recorded. The high resolution C1s XPS spectra as shown in Fig. 6b reveals the presence of three chemically different carbon in the structure of cellulose. The three peaks at 284.8, 286.5 and 287.7 eV could be attributed to chemically different carbon, one with ether link, second carbon linked to hydroxyl groups and third carbon having two ether links, respectively (Aggrawal et al. 2019). The high resolution O1s spectra (Fig. 6c) reveals the presence of two chemically different oxygen attributed to hydroxyl and glycosidic oxygen present in the structure of cellulose at 531.3 and 532. The third oxygen present in very less amount may arise due to environmental errors. The high resolution Cu2p spectra is shown in Fig. 6d. It confirms the presence of Cu in +2 oxidation state as the XPS peaks observed at 935.5 eV and 954.2 eV for Cu 2p<sub>3/2</sub> and Cu 2p<sub>1/2</sub>, respectively along with the satellite peaks at 943.3 eV and 962.7 eV matches well with the reported values in literature (Cao et al. 2019). Hence the immobilization of CuO in the paper matrices have been confirmed by XPS analysis.

The antimicrobial activity of the synthesized CuO immobilized paper matrices have been carried out by deactivating both fungus and Gram negative bacterium. The antibacterial activity has been studied using colony count method and summarized in Table S4. It has been observed that 30CuOP specimen has deactivated the complete *E. coli* count of  $2.8 \times 10^7$  within 2 h of visible light exposure. When compared

with the blank paper (specimen P), almost no reduction in bacterial count was estimated, whereas 40, 50, 95 and 99.99 % reduction in *E. coli* count has estimated in the specimens 5CuOP, 10CuOP, 15CuOP and 20CuOP, respectively. With an increase in the content of CuO in the paper matrices, there is an improvement of the antibacterial activity. The antibacterial activity has been induced by the interaction of CuO nanoflakes with the cell membrane of the *E. coli* which resulted in the inhibition of active cell transport process, thus induces the cell lysis (Sharmila et al. 2016; Sirelkhatim et al. 2015; Safaei et al. 2019; Villanueva et al. 2016). It also leads to damage of biochemical metabolisms of the bacterial cell. Another possibility of antibacterial mechanism is the generation of ROS species which further deactivates bacterial cells (Maheswari et al. 2017). Further, the experiment has been carried out in triplicate and standard deviation has been calculated in the log reduction values of bacterial colonies and given in Fig. 7. Almost similar results have been observed while carrying out the experiment for multiple times.

Furthermore, antifungal activity has been studied using zone of inhibition method and results has been summarized in Table 1. The antifungal activity has been carried out for 28 days and it has been observed that specimen 30CuOP remain intact even after 28 days of fungal infection (Table 1). The brown colour of the paper is intact in case of specimen 20CuOP until 15 days of infection. Whereas, specimen P and 5CuOP has been completely damaged by the fungal cells. The cellular wall of the fungus is composed of polysaccharide compounds and glycoprotein. The CuO nanoflakes may get attached to the wall of the fungus which interrupts its diffusibility and damage the cell membrane. Another reason might be the development of ROS, disruption of cellular homeostasis and dynamic equilibrium. It leads to leakage of cell contents resulting in death of fungal cell (Safaei et al. 2019). Hence, the increase in content of CuO in paper matrices has resulted in the increase in antibacterial activity as that of antibacterial activity. The material has shown promising results against both bacterial and fungal cells.

## Conclusion

In summary, an ultrafast (5 min only) process has been developed to immobilization CuO nanoflakes on the surface of the cellulose fibers of paper matrices by a microwave assisted method. Neither linker, binder nor retention aid has been used for the immobilization, which sustain for five consecutive recyclability cycles. All these specimens have shown good antimicrobial activity by deactivating a Gram negative bacterium, *E.coli* and paper eating fungus, *G. trabeum*. Among all these specimens, 30CuOP has shown best antimicrobial activity against both bacteria and fungus. The concept of ultrafast immobilization of CuO nanoflakes on the paper matrices and phenomenal antimicrobial activity has not only academic values but could also open up industrial revenues in medical and water purification.

## Declarations

### CONFLICT OF INTEREST

The authors declare no competing financial interest. There is no animal studies or human participants involvement in the study.

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## Table

Due to technical limitations, Table 1 is available as a download in the supplementary files.

## Figures

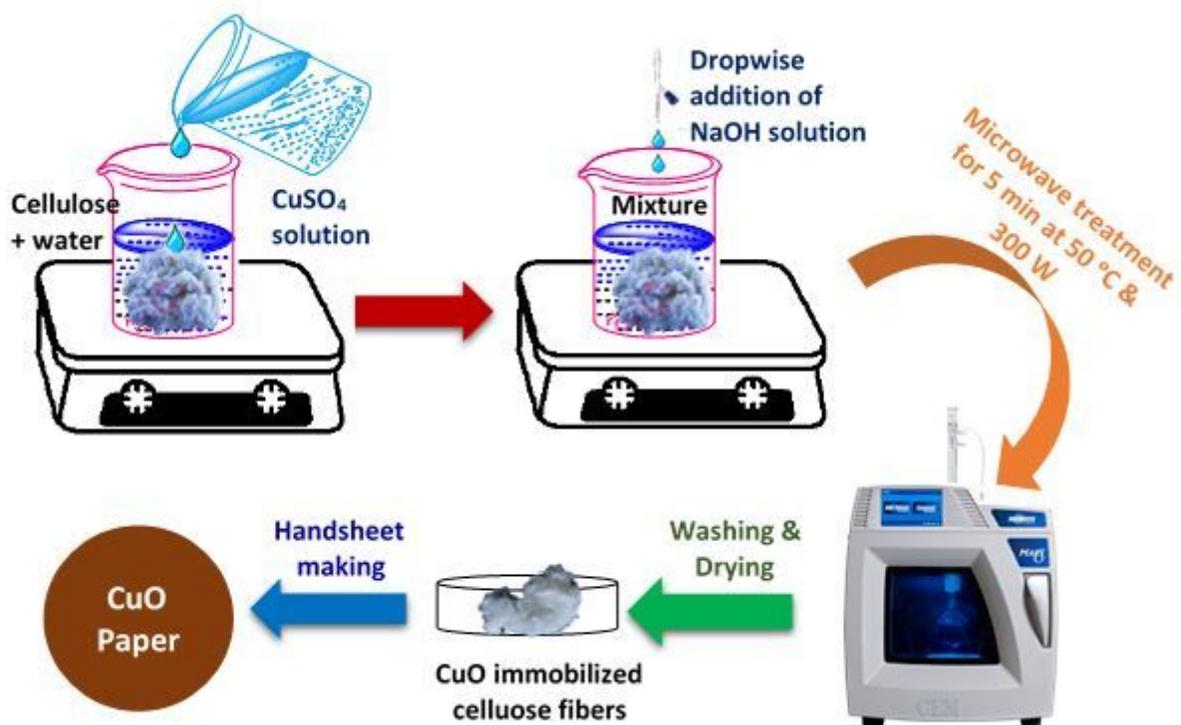


Figure 1

Schematic representation for the ultrafast immobilization CuO nanostructures on the paper matrices using microwave assisted method.

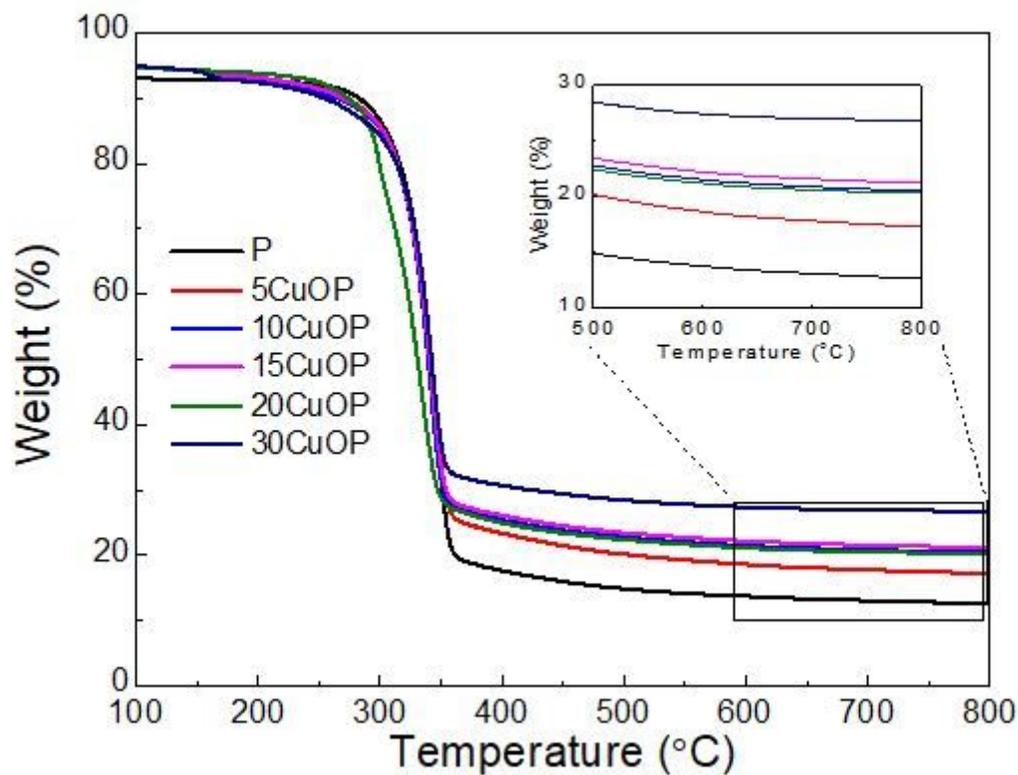


Figure 2

TGA curves of CuO immobilized paper matrices carried out in argon atmosphere.

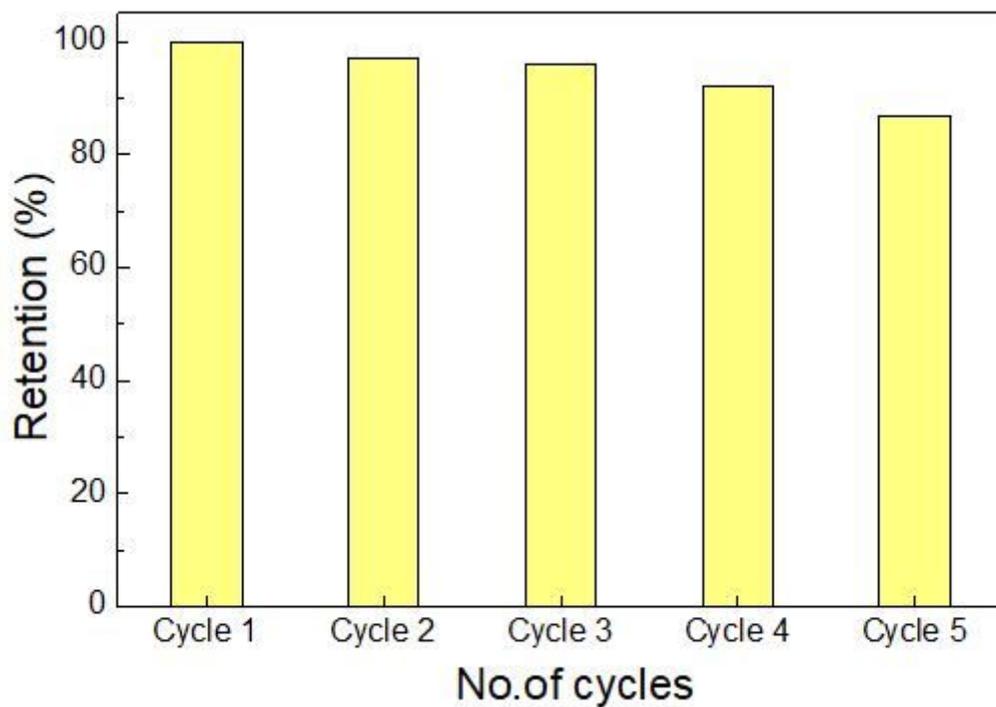
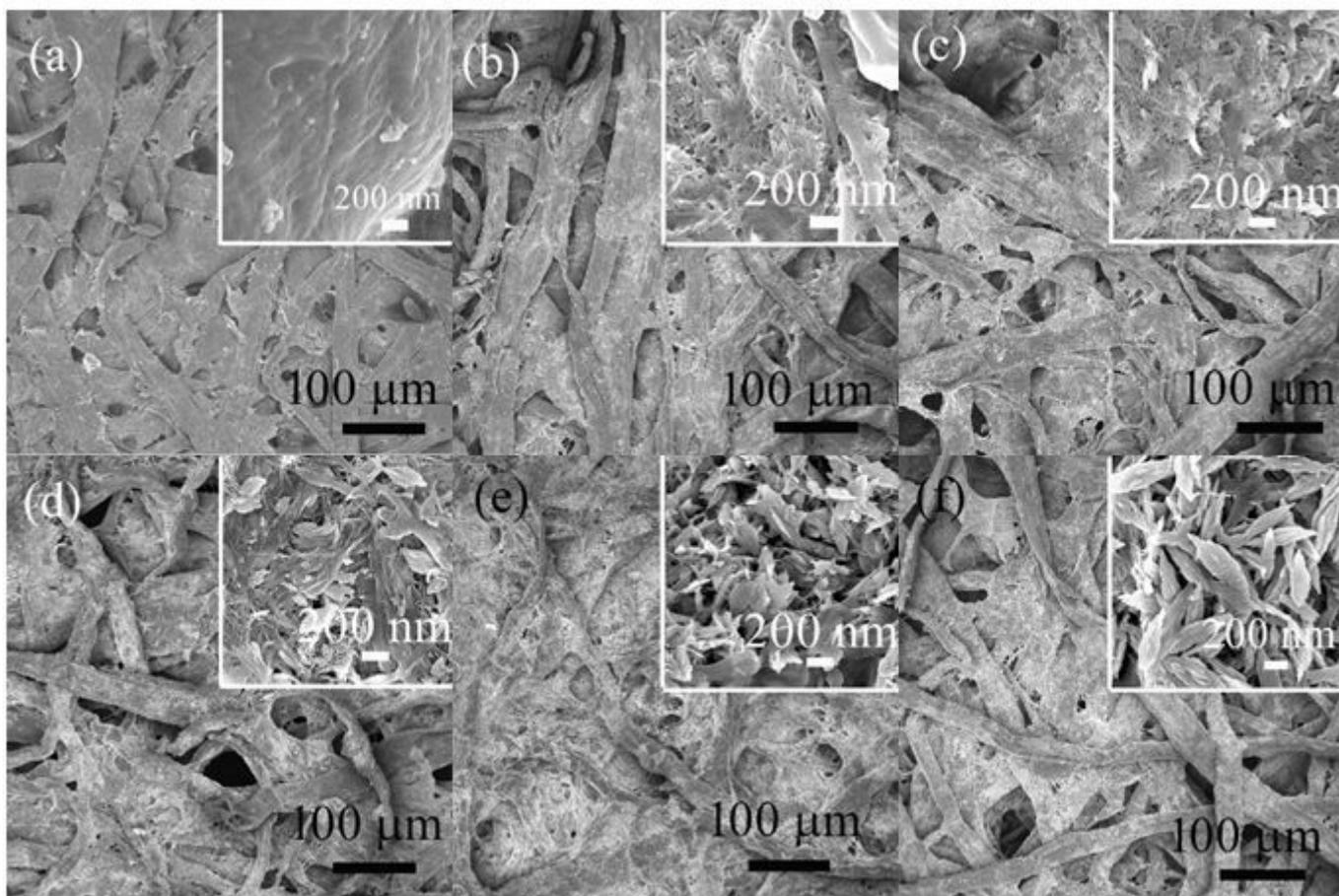


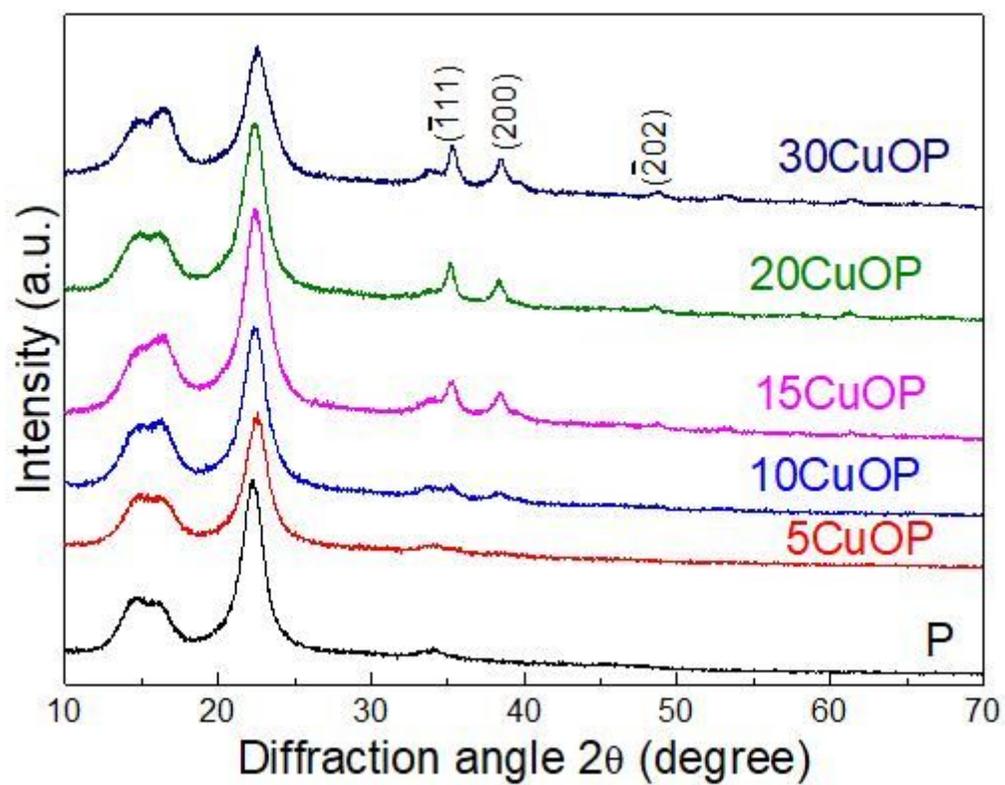
Figure 3

Retention of CuO in paper matrices after five consecutive cycles of pulp dispersion and paper fabrication.



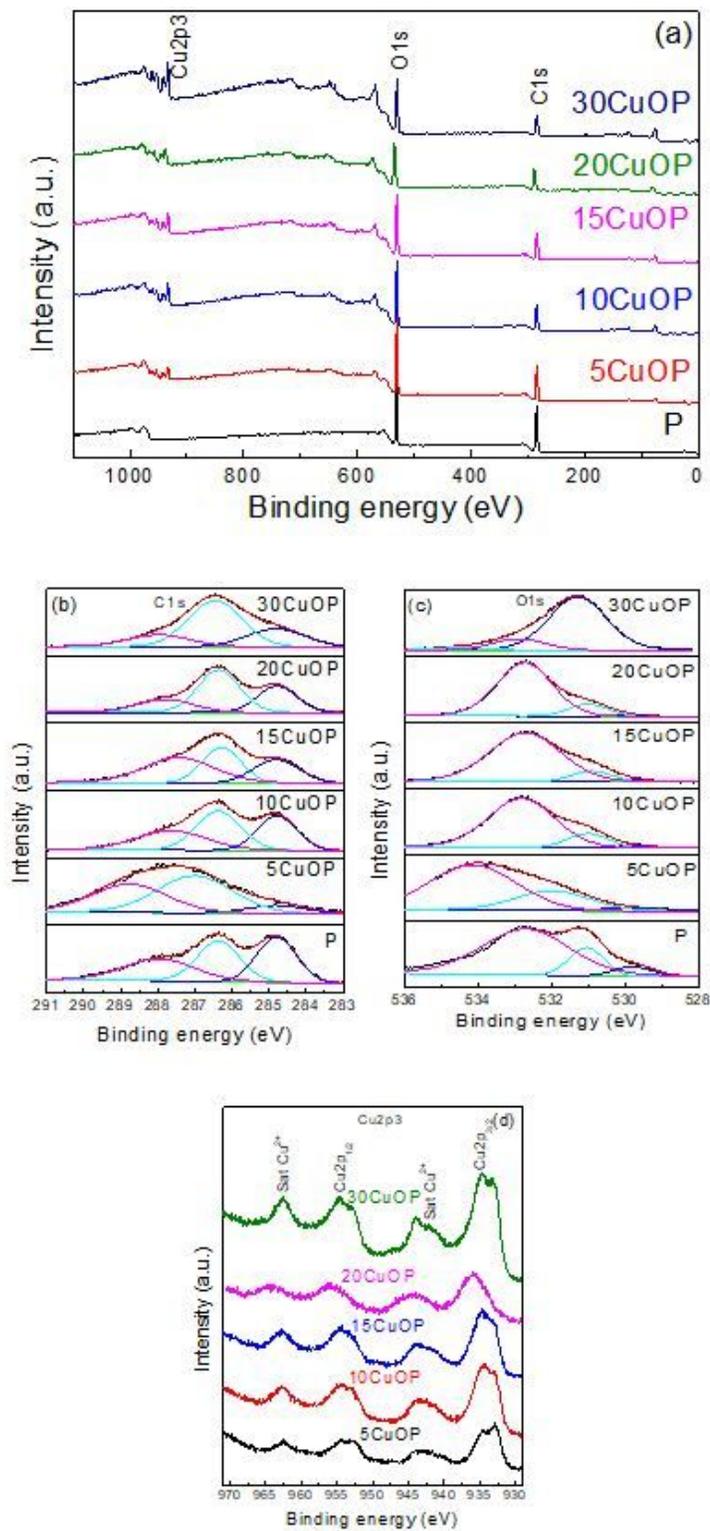
**Figure 4**

The FESEM images of CuO nanostructures immobilized paper matrices with varying CuO content: a) P, b) 5CuOP, c) 10CuOP, d) 15 CuOP, e) 20CuOP and f) 30CuOP. The inset shows high magnification image respectively.



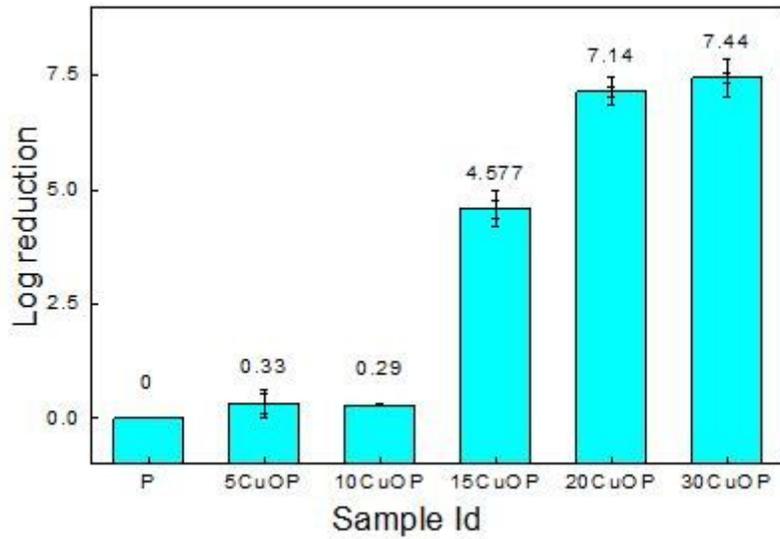
**Figure 5**

X-ray diffraction patterns of CuO immobilized paper matrices.



**Figure 6**

XPS spectra of CuO immobilized paper matrices: a) Su1s, b) C1s, c) O1s and d) Cu2p.



**Figure 7**

Reduction in *E. coli* growth after 2h of visible light exposure with standard deviation.

## Supplementary Files

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