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Biocompatible nano hydroxyapatite – curcumin bi-coated antibacterial activated carbon for water purification

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Activated Carbon has been used for water purification since ancient times due to its well-known sorbent properties. However it is not capable of disinfecting water borne pathogens such as bacteria. The main objective of this study was to incorporate antibacterial properties while maintaining the existing properties of Granular Activated Carbon (GAC). This was achieved by a biocompatible double coating on to GAC which consists of Hydroxyapatite (HAP) nanoparticles and on top of those curcumin molecules. Coating of GAC with HAP was carried out using in-situ precipitation of HAP under basic conditions and obtained hydroxyapatite coated GAC (HAP/GAC). A layer of curcumin molecules were then attached on top of the HAP coating in order to obtain HAP-Curcumin bi-coated GAC (HAP/C/GAC). Synthesized HAP/GAC and HAP/C/GAC were characterized using FT-IR spectroscopy, Scanning Electron Microscopy, X-ray Diffractometry and Thermogravimetry (TGA). Characterization revealed that needle shaped HAP nanoparticles (50 - 100 nm in width and around 200 - 500 nm in length) can be anchored and immobilized successfully on GAC which in turns enhances the adhesion of curcumin on it. Antibacterial properties on pure GAC, HAP/GAC, HAP/C/GAC was then investigated using both gram negative (*Escherichia coli*) and gram positive (*Staphylococcus aureus*) bacteria. The results showed that the antibacterial properties of HAP/C/GAC is remarkably higher than that of HAP/GAC and the antibacterial activity of pure GAC was negligible.

Introduction

Planet Earth is often called "blue planet"[1] due to its vast surface covering of water. But in spite, the amount of water that is available for drinking is only less than 2.5%[1] being the remaining is either inaccessible or inconsumable.[1] Apart from the above fact, a considerably large portion of potable water gets polluted as a result of human activities[2] while the usage of fresh water in agriculture and industries further limits its availability for consumption[3] exacerbating the situation. Therefore scarcity of clean and safe drinking water has become a crisis which beseeches attention.[4] Moreover it has been identified that about 10% of the diseases that jeopardizes global population can be prevented if drinking water, hygiene and sanitation is improved.[4] The basic idea of purifying water is to remove turbidity, other chemical and microbial contaminants in potable water sources.[5] Activated carbon is a widely used purification agent due to its well-known role as a sorbent, low cost abundance and environmental benignness.[6] Also it is capable of improving the quality of drinking water by removing dust, sand, rust, color, objectionable tastes and odors.[7] However early studies have shown that there's no significant difference between water filtered through activated carbon and unfiltered tap water with respect to the consistence of microbial contaminants in them.[8] Therefore, often a separate disinfector like chlorine,[9] ozone[10] or UV is used in filtration apparatus where activated carbon is present as the matrix.[11] On contrary, these disinfector agents are more likely to form harmful DBP's (Disinfection by-products)[12] such as trihalomethanes, haloaceticacids and aldehydes[13] whenever it's used in water treatment due to high oxidative capacity.[12,13]

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Consequently in continuing their use, we often have to compromise between the effectiveness of disinfection and the formation of harmful DBP's.[12] Even though nanotechnology promises to give solutions for this dilemma, it has also being recorded that for a nanomaterial to be an effective and reliable purifier, these particles should be anchored thoroughly onto packaging material such as GAC.[12] This dilemma pulls the attention towards inventing disinfectants which are not strong oxidants in which case nanotechnology appears to be a promising relief.[12]

As for nanomaterials, Silver nanoparticles,[12,14] metal oxide nanoparticles such as TiO_2[12] and ZnO,[12] fullerenes[12] and carbon nanotubes[12] are reported in water purification as non-DBP producers.[12] However silver nanoparticles are recognized to cause intoxication in humans leading to various health issues.[15] High water solubility of ZnO brings down the opportunities for it in water treatment.[12] Furthermore concentrations >5 mM of ZnO nanoparticles have reported to reduce human T cell viability.[16] The susceptibility of coagulation of fullerenes by the salts present in water, and the toxicity to mammalian cells[12] limits its audience in water treatment grounds.[17]

HAP is a calcium phosphate ceramic material with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$ and nano HAP is a well-known material for removing heavy metal ions from water. HAP nanoparticles have become imperative tools in medical industry due to its high biocompatibility with human physiology and has made it one of the best choices in drug and gene delivery.[18,19,20] Also it has been reported in antibacterial applications, as a surface for adhesion of silver.[21] Apart from that, it's evident that HAP/GAC nanocomposite has high heavy metal sorption capacity.[22]

On the other hand Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a hydrophobic polyphenol, a natural compound extracted from the rhizome of plant *Curcuma longa* (turmeric). It has been in use for more than decades in Ayurvedic medicine and Asian cuisine[23] due to its antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities.[24] Curcumin has well proven antibacterial properties through inhibiting the bacterial endotoxin induced cytokines secretion and related processes thereby directly suppressing the bacterial cell growth.[25, 26]

The main objective of this study is to develop a multifunctional biocompatible GAC based filtering matrix with enhanced anti-bacterial properties.

In our investigation, GAC was selected as a matrix material. By keeping inherent properties of GAC intact and to overcome the lacking antibacterial property, well known antibacterial materials such as HAP and Curcumin were introduced to GAC.

Coating of HAP nanoparticles on to GAC was carried out by modifying our previously reported procedure.[22] This HAP layer was further functionalized with Curcumin molecules to develop HAP-Curcumin bi-coated GAC. And these two compounds were then tested for their antibacterial activity against *E. coli* and *S. aureus* as gram negative and gram positive bacteria respectively.

Experimental Section.

Materials and Methods

Granular Activated Carbon (coconut shell) and Curcumin (80%) were purchased from Sigma-Aldrich Ltd. Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), minimum assay 98% from Techno Pharmchem, India was used in the synthesis along with Sodium dihydrogenphosphate dihydrate ($NaH_2PO_4.2H_2O$) of BDH Chemicals and Ammonia solutions were prepared from 25% pure NH_3 from MERCK, Germany. All these chemicals were of reagent grade, and double distilled water was used throughout. All the solid reactants and synthesized nanoparticles were weighed in the analytical balance (CAS- CAY 120). Drying was done using the electric oven (National, Japan) and pH of the solutions were measured using pH meter (EUTECH – pH 510).

Synthesis of HAP coated GAC (HAP/GAC). The synthesis was done by mixing ($Ca(NO_3)_2.4H_2O$) with $NaH_2PO_4.2H_2O$ in 1.67 Ca/P molar ratio in 300.0 ml of double distilled water and heated with vigorous stirring. When the solution reached 85 °C, 300.0 ml of 25% NH_4OH and GAC (12.0 g) was added at once and the mixture was stirred for another 2 hours maintaining the temperature at 85 °C. In two hours time, stirring was discontinued and the mixture was aged at 85°C for 24 hours. The mixture was then allowed to cool under the room temperature and solid HAP/GAC were allowed to settle at the

bottom. After decanting the overlying solution, HAP/GAC were washed with double distilled water until the pH of the washings become pH 7. The resultant mass was filtered and oven dried at 80-100°C for 3 hours.

Synthesis of HAP-Curcumin bi-coated GAC (HAP/C/GAC). Nano composites synthesized as described above, were further coated with Curcumin following the same procedure. Curcumin was added in 1:1 molar ratio with HAP prior to the addition of NH_4OH and GAC. Photographs showing the color changes during synthesis of (HAP/C/GAC) are given in supporting materials.

Characterization Techniques. The presence of functional groups of Curcumin on HAP layer was confirmed using Fourier Transform Infrared Spectrometer AVATAR-320 (Thermo Nicolet). The FT-IR spectra were obtained over the region 400-4000 cm^{-1} using KBr pellet technique. Pellet was prepared by mixing 2.0 mg of the sample with 200.0 mg of oven dried spectroscopy grade KBr (Sigma-Aldrich). The presence of Curcumin was further confirmed by carrying out TGA analysis. The TGA analysis was performed using nearly 10.0 mg in Thermo-gravimetric Analyser (SDT Q600). The temperature range used for the analysis was 25 - 1000 ºC. The surface morphology and microstructural features of synthesized nano composites were studied using Scanning Electron Microscope (HITACHI SU6600). X-Ray diffractometry was carried out on a Bruker D8 Focus X-ray powder Diffractometer using CuKα radiation (λ = 0.154 nm) over a 2Θ range of 3-60^o, with a step size of 0.02^o and a step time of 1 s.

Microbiological Techniques.

Lysogenic broth (LB) Agar media were purchased from the manufacturer Hardy Diagnostic and used as the medium for bacterial growth. Medium preparation was done by dissolving 10.0 g of LB and 2.5 g of Agar in 500.0 ml of double distilled water. Inhibition zone study was done taking Gentamicin (Mycin®, NEON laboratories Ltd.) as the positive control and double distilled water as the negative control.

All the microbiological work was performed at the Laminar hood (BioBase). Bacterial growth media were autoclaved in Autoclave machine (ALP Co. Ltd. Model KT – 305D -230v, 50/60 Hz) before use and all the glassware including petri plates were incubated at 180°C for 2 hrs in oven (Memmert Beschickung loading model 100 -800) before use. Bacteria inoculated plates were incubated at 37 °C in incubator (Memmert Beschickung loading model 100 - 800) overnight for growth. For the sonication purposes sonicator (Sonorox super RK 1028 CH, BANDELIN Electronics, Berlin) was used. For the bacterial cell counting, Haemocytometer (Neubauer, Germany) was used together with the optical microscope (OLYMPUS CK X41).

Disc Diffusion Assay. Sterilized paper discs were loaded with 10 µl of sonicated nanoparticle suspensions (HAP, HAP coated with Curcumin) and were mounted on E.coli spread plates. Nanoparticle suspensions were prepared by dissolving 0.1 g of nanoparticles in 10.0 ml of autoclaved distilled water. Prepared suspensions were sonicated for 15 min before use. 10 µl aliquot of 1000 ppm Gentamicin was used as the positive control. The plates were then incubated at 37 ºC for 24 hrs and then observed.

Column Technique. Suspensions of *E. coli* and *S. aureus* were prepared in autoclaved double distilled water. All the filter materials were autoclaved before use. Autoclaved pure GAC, HAP nanoparticles and the two nano composites: HAP/GAC and HAP/C/GAC were loaded into separate syringes to form mini-water filters with 5 mm diameter and 2.5 cm length. Double distilled water was passed through each filter for 2 minutes prior to the suspension at a rate of 0.03 ml/ min. Then the bacterial suspension was allowed to drain through the filter in the same rate under the laminar hood.

Filtered water was collected into autoclaved Pyrex tubes and was kept in an ice bath to inhibit bacterial multiplication. Bacterial contents of these water samples were analysed qualitatively using impregnation methods: by seeding 50.0 ml of autoclaved LB Agar media with 200.0 µl of the filtered water samples. The capacity of prolonged antibacterial efficacy of nano composites were analysed by draining bacterial suspension over 5 minutes and the resultant filtered water samples were analysed for the bacterial content using the same procedure. Quantitative analysis was done using the haemocytometer and spread plate technique.

Results and Discussion

Characterization of HAP, HAP/GAC using FT-IR spectrophotometry. HAP nanoparticles were synthesized on GAC as given in the experimental section. The presence of HAP coating on GAC was

characterized using FT-IR spectroscopy. The FT-IR spectra obtained for pure GAC, pure HAP and HAP/GAC are given in the Figure 1.

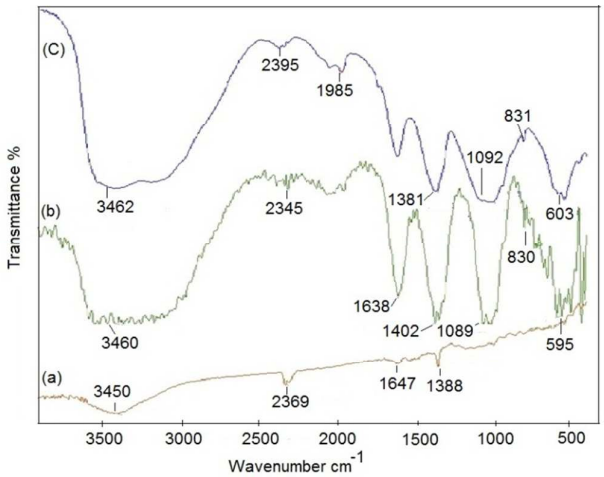


Figure 1. FT-IR spectra of pure (a) GAC (b) HAP/GAC (c) pure HAP

As seen in the Figure 1, FT-IR spectrum of HAP/GAC and FT-IR spectrum of pure HAP are very much similar to each other. In both HAP/GAC and pure HAP, the broad peak that appears at 3000-3500 cm^{-1} region accounts for the stretching vibration mode of H bonded OH groups present in HAP[27]. Peak at 1092 cm^{-1} corresponds to the stretching mode of PO_4^{3-} groups and the ones at 603, 831 cm^{-1} are the bending modes of PO_4^{3-} groups.[27] The absence of broad band, at 2600-3000 cm^{-1} region and, peaks at 1600 cm^{-1} and at 875-880 cm^{-1} which of those that are characteristics of amorphous calcium phosphate, carbonate apatites and Ca deficient HAP respectively, indicates that these contaminant compounds are not formed during the HAP nanoparticle synthesis.[28] Therefore this assures the presence of pure HAP nanoparticles on GAC. With compared to pure GAC spectrum, the HAP/GAC spectrum shows a broad band at 3000- 3500 cm^{-1} indicating the increased amount of OH groups on GAC as a result of coating it with HAP.

Morphology and nature of coating of HAP, HAP/GAC using SEM. The morphology and the size of HAP nanoparticles synthesized on GAC was determined using scanning electron microscopy. The SEM micrographs obtained for pure HAP and HAP/GAC are given in the Figure 2.

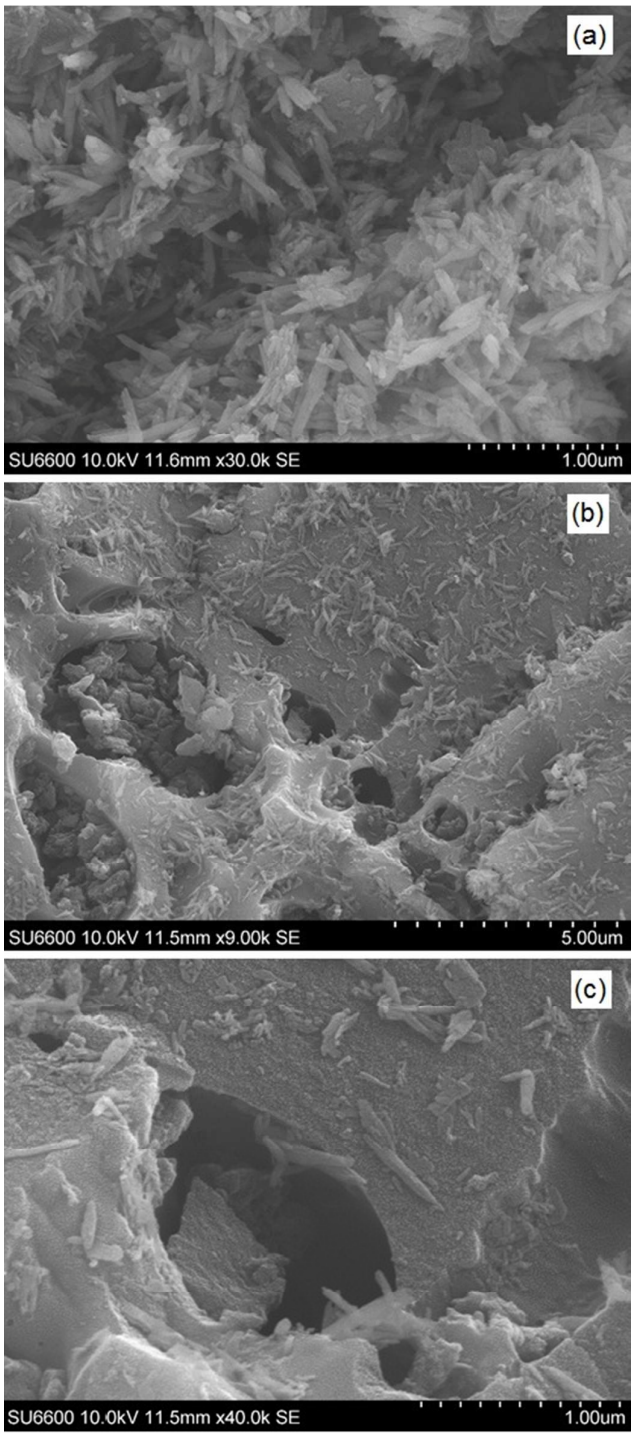


Figure 2. SEM images of (a) pure nano Hydroxyapatite (HAP) (b) HAP/GAC low magnification (c) HAP/GAC high magnification

Figure 2 (a) shows the nearly needle shaped HAP nanoparticles with the aspect ratio of nearly 50 - 100 nm in width and around 200-500 nm in length.

The SEM image obtained for HAP/GAC (Figure 2(b) and (c)) shows that the coating of HAP on GAC is not even as previously observed[22] and instead, the needle shaped particles are scattered on the GAC surface permitting most of the GAC surface to be exposed. This observed change in coating may be attributed to the addition of ammonia solution at once, during the synthesis of HAP on GAC thereby allowing fast nucleation and quick separation between nucleation and nanoparticle growth leading to a monodispersed nanoparticle formation as suggested by LaMer model.[29] According to Figures 2(b) and 2(c), almost all the micropores of GAC are uncovered with HAP and this can be considered as an advantage since most of the adsorption occurs mainly at the pore sites.[30] The image also reveals that HAP nanoparticles are held well in the porous matrix of GAC allowing a successful coat. It can also be suggested that the HAP nanoparticles are held strongly by the carbon matrix as calcium leaching was not observed for the leaching test carried out.

Characterization of HAP, HAP/GAC using XRD. Further confirmation on this coating is revealed in the XRD pattern of the HAP coated GAC which is shown in Figure 3. The comparison given with standard HAP peaks shows that synthesized nanocomposite comprises HAP nanoparticles that are in compliance with the standard hexagonal crystal system of HAP and the absence of other contaminants. The Peaks at 2Θ positions of 26º, 29°, 32°-34°, 40°, 46°-54° are in good agreement with the previously reported crystalline HAP.[31,32] This shows that our synthesized HAP was nano sized with crystalline structure. The broad nature observed specially at the 2Θ regions 26 and 40 are in good agreement with the presence of GAC matrix on which crystalline HAP has been coated. Also sharp XRD peaks for HAP present on GAC matrix account for its high crystallinity in contrast to the previously established data by our group.[22]

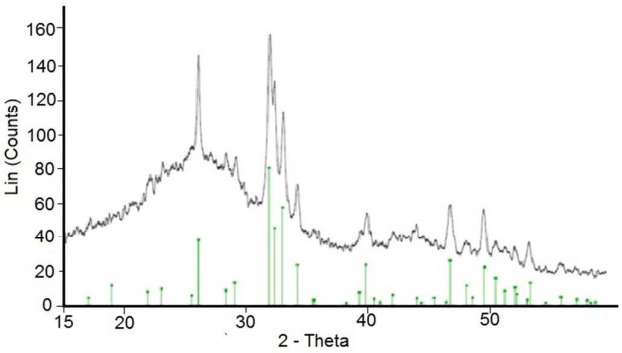
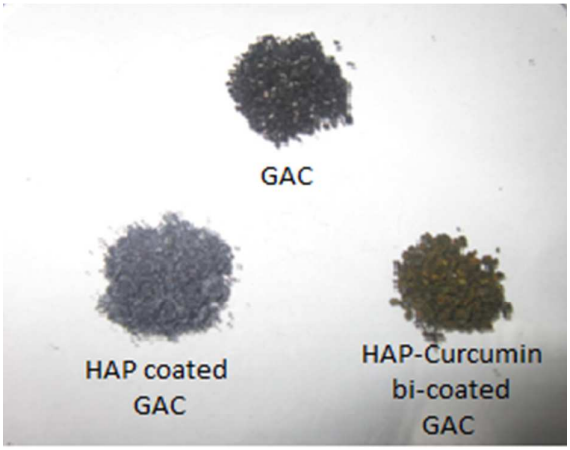


Figure 3. Comparison of XRD pattern of HAP/GAC with standard HAP

Characterization of HAP/C/GAC using FT-IR spectrophotometry. HAP coated GAC nanocomposite was further functionalized by coating it with Curcumin molecules as given in the experimental section. The physical appearance of GAC, HAP/GAC and HAP/C/GAC is given in the Figure 4 . HAP/GAC has a whitish touch in its color than uncoated GAC. The presence of Curcumin on the HAP layer is



very clear due to the yellow color appeared on HAP/C/GAC. This was obtained after washing the HAP/C/GAC with plenty of water until the absence of peak at 1628 cm^{-1} in FT-IR[33,34] and 435 nm peak in UV-Vis[34] which corresponds to the curcumin.

Figure 4 . Comparison of physical appearances of synthesized Nano composites with GAC

HAP-Curcumin bi-coated GAC was subjected to FT-IR spectroscopy and the spectra obtained for three samples namely, HAP/C/GAC, pure Curcumin and HAP/GAC were compared in the Figure 5.

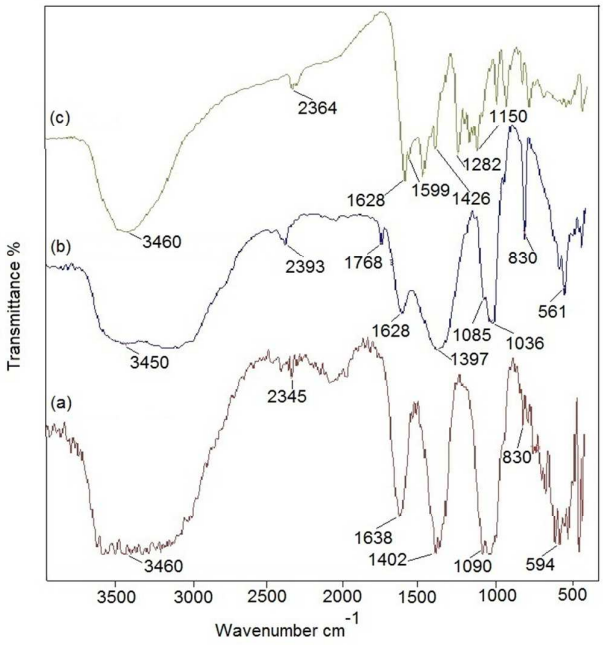


Figure 5. Comparison of FT-IR spectra of (a) HAP coated GAC (b) HAP-Curcumin bi-coated GAC (c) Curcumin

The spectrum of Curcumin in Figure 5 contains the broad band around 3500 cm^{-1} which is an indication of its H bonded phenolic OH group. The peak at 1628 cm^{-1} is due to the aryl substituted C=C bond.[34] The shoulder appearing on to its right at 1599 cm^{-1} accounts for the conjugated C=C bonds of the aromatic ring. The peak at 1150 cm^{-1} stands for the C–O bond of the ether group (OCH_3).[35] These values are in accordance with the FT-IR peaks of curcumin reported previously.[36] When comparing the three spectra together the middle spectrum (HAP/C/GAC) has similarities to both HAP/GAC as well as to pure curcumin. The broad peak present in the region of 3000 cm^{-1} - 3500 cm^{-1} indicates the presence of both HAP coating as well as curcumin. However, the characteristic band of curcumin's carbonyl group seen at 1628 cm^{-1} present on HAP/C/GAC is absent on HAP/GAC. This is evident of having a successful coating of curcumin on top of the HAP coating. It was also confirmed that curcumin coats specifically onto HAP layer and not onto the carbon matrix. For this we carried out an experiment to coat curcumin onto pure GAC and pure HAP using the same coating procedure under HAP/C/GAC. The FT-IR spectra obtained for these experiments, (given in the supporting materials) support the absence of curcumin on pure GAC. This selective adhesion of curcumin onto HAP can be attributed to the metal ion chelating ability of curcumin which ensures its binding to HAP via HAP's Calcium ions.

Characterization using Thermo Gravimetric Analysis. Synthesized nano-composites and their precursors were subjected to TGA analysis and the resulted thermograms are shown in Figure 6.

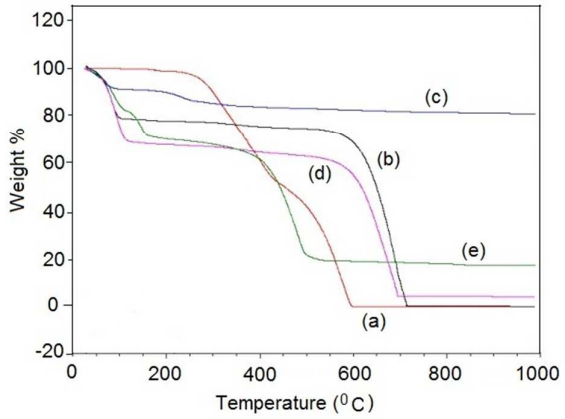


Figure 6 . Comparison of TGA curves obtained for (a) Curcumin (b) GAC (c) HAP (d) HAP/Curcumin/GAC (e) HAP/GAC

As seen in the figure 6, it is evident that two of the main precursors present in synthesized nano-composites are not thermo-stable up to 1000 °C. GAC and Curcumin has burned under air leaving no residue during 600 – 800 °C. On contrary, HAP has shown a significant stability towards temperature. The first endothermic region of HAP's TGA curve corresponds to the removal of physically adsorbed water from the crystal lattice.[32] Furthermore, absence of any other endothermic regions assure the absence of other contaminant compounds like $CaHPO_4$, $Ca(OH)_2$ thereby confirming the purity of HAP.[37] Also it's visible that both the nanocomposites maintain a non-zero mass when 1000°C is achieved. This is a clear evidence that these two nano composites contain HAP nanoparticles in them which withstands the higher temperatures.

Determination of antibacterial activity of synthesized nano-composites.

Nano composites containing GAC, HAP and HAP/C were subjected to antibacterial activity using disc diffusion assay. Gentamycin (10 µg/ml) was used as the positive control and water as the negative control and the results obtained are shown in Figure 7. The activity of each material is expressed as the diameter of growth inhibition area in cm.

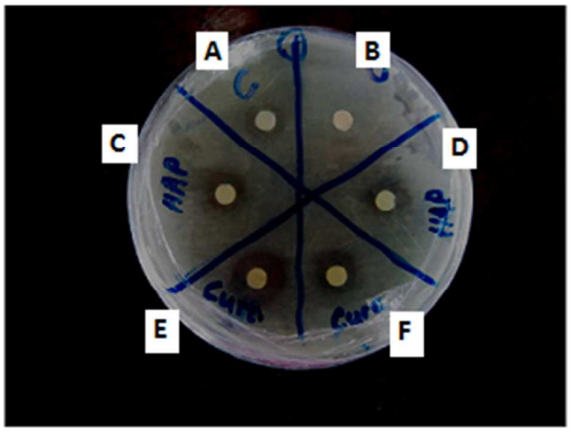


Figure 7. Inhibitions zones. A – Positive Control (1.2 cm); B – Negative Control (0.0 cm); C,D – HAP (1.2 cm, 1.1 cm); E,F – HAP coated with Curcumin (1.5 cm, 1.3 cm)

Compared to the positive control Gentamycin (10 µg/ml), HAP and HAP/C (100 µg/ml) showed similar level of antibacterial activity against *E. coli.* These results clearly show that nano composites containing HAP and HAP/C have an effective antibacterial activity with the highest in HAP/C.

The ability of synthesized HAP/GAC and HAP/C/GAC nano-composites in removing bacteria in water, has been experimented using a known amount (5.2 x 10^9 CFU/ml) of *E.coli* and *S.aureus* bacterial suspensions. This bacterial suspension was passed through columns prepared with HAP/GAC and HAP/C/GAC. The filtrates were analyzed using impregnation method. The results obtained are given in the Figure ~~9~~ 8 .

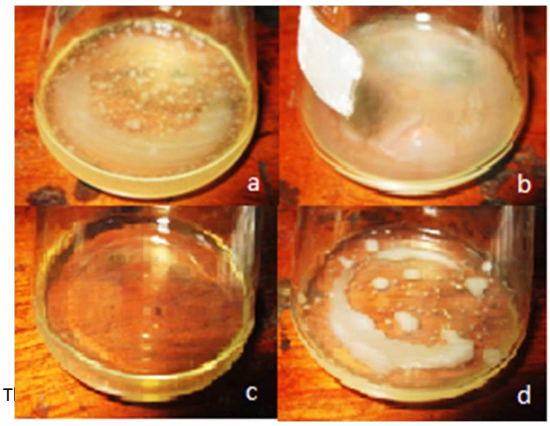


Figure 8 (a) Bacterial suspension used for filtration (b) Filtrate of GAC filter (c) Filtrate of HAP filter (d) Filtrate of HAP/GAC filter

According to the results highest number of bacterial colonies is present in the filtrate of uncoated GAC filter. This is in accordance with the reported data where it mentions the formation of biofilm on GAC can facilitate the multiplication of coliforms rather than reducing their number.[1] In general both the nanoparticle containing filters seemed to have reduced the bacterial contamination in water. However no bacterial colonies can be found in water samples that were passed through the columns containing pure HAP. Antibacterial activity observed with HAP/GAC nanocomposite is in between pure GAC and pure HAP. And it is evident that the antibacterial properties of synthesized HAP are retained even if they are immobilized by coating on to GAC. Therefore the aim of this study was successfully achieved.

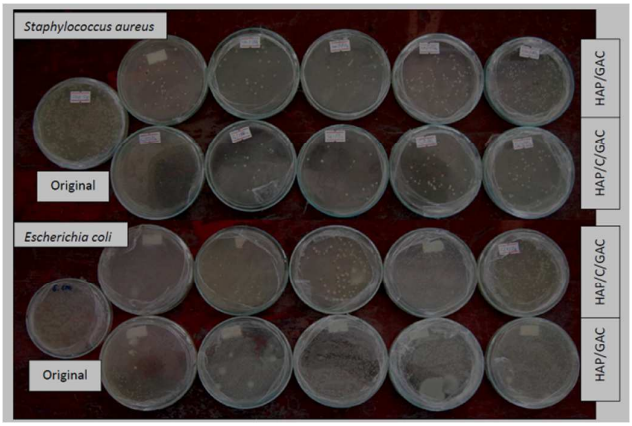


Figure 9. Antibacterial Activity against *E. coli* and *S. aureus*

The extended experimental studies on antibacterial activity of synthesized filter materials namely GAC, HAP/GAC and HAP/C/GAC were carried out in order to compare the antibacterial properties of HAP/C/GAC to pure GAC. The results obtained are shown in the Figure 9.

In this experiment antibacterial activity was demonstrated against number of colony forming units. Compared to the number of colonies present in the original sample, the number of colonies found in HAP/GAC filtrate and HAP/C/GAC filtrate are extremely low for the case of *S. aureus.* A close observation shows that absolutely no colonies are found in the 1st ml for HAP/C/GAC as well as in HAP/GAC column. In HAP/GAC, a similar number of colonies are observed in the 2nd and 3rd ml followed by a slight increment in the 4th and 5th ml. In HAP/C/GAC, similar number of colonies are there in 2nd and 3rd ml and slight increment is observed up to 5th ml. However, compared to HAP/GAC the number of colonies are significantly low and this can be attributed to the presence of Curcumin.

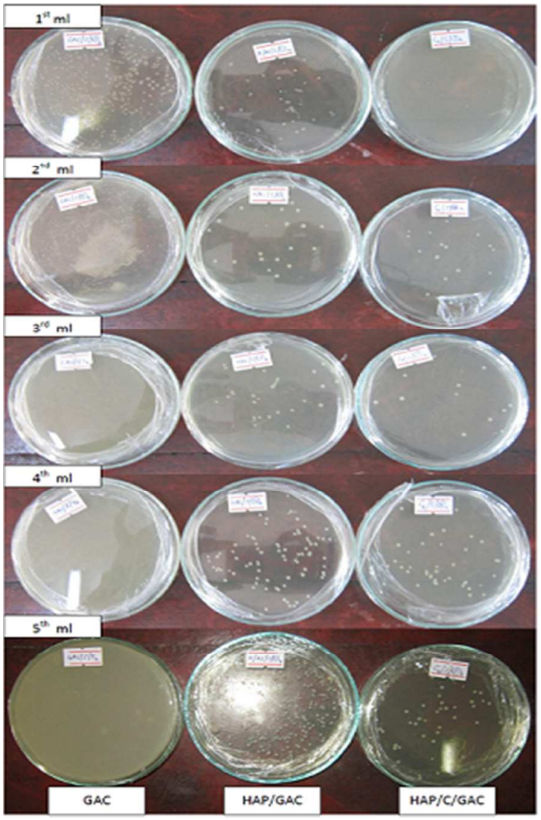


Figure 10. Comparison of the effects of HAP/GAC and HAP/C/GAC filters with the GAC filter.

A spread plate technique was carried out for filter systems containing pure GAC, HAP/GAC and HAP/C/GAC in order to compare their activity against *S. aureus.* The results are given in the Figure 10 . As seen in the figure, the amount of growth of bacteria in the filtrate of pure GAC has given rise to a lawn of bacteria in the spread plate and the thickness of the lawn has increased when going from the 3rd towards 5th. The reason can be the same that was mentioned before, facilitation of the growth of bacteria by the filter bed rather than a reduction.

Nevertheless in both nanocomposite containing filters, the viable cell count in the filtrate has tremendously decreased compared to the pure GAC filter. The filtrate obtained from HAP/C/GAC shows absolutely no growth for the first fraction. Further, it is clear that the filtrate obtained from HAP/C/GAC for 2nd and 3rd fractions are having only a very few and similar number of colonies with the slight increment going from 4th to 5th. However with compared to the HAP/C/GAC the amount of colonies at the HAP/GAC has a significant increment in number. On the other hand, the gradual reduction of the antibacterial activity observed in both filters may be due to the saturation of the activity sites with bacteria due to the repeated use of filter. Therefore further studies should be done to get a clear idea about the capacity of the filters towards bacterial disinfection.

Conclusion

A novel biocompatible granular activated carbon nanocomposite was synthesized by immobilizing HAP nanoparticles and curcumin on GAC to develop a filter material in water purification applications. This will successfully overcome the health issues related to the release of metal and metal oxide nanoparticles to water during purification process using current metal and metal oxide based anti-bacterial water filters. Furthermore, HAP-curcumin bi-coated GAC carries a significant capacity to purify contaminated water with the antibacterial properties experienced by the presence of both curcumin and HAP. The novel material will be developed as a point-of-use filter material to be used in rural areas in Sri Lanka. The possibilities of regeneration of the filter material are also in progress.

Acknowledgements

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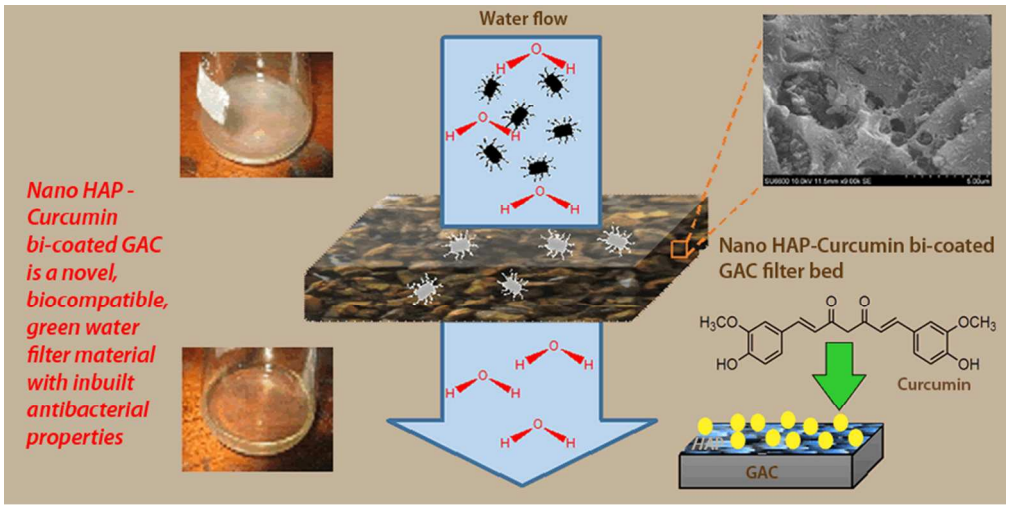

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