6.1. Introduction

Minute observation of natural phenomena’s can sometimes become a great source of ideas and inspiration for the development of wonderful technologies. Many advanced technologies of human civilization are the outcome of mimicking the nature. This mimicking nature has also been reflected by the scientific reports coming out from the research and developments going on worldwide in the past decades\textsuperscript{160-164}. In this context, a new idea is going to be introduced to develop a kind of highly absorbent, stable, robust cotton patch following the mechanism by which a silk-worm makes its cocoon. The cocoon is composed of the fibroin protein fibers coated with another kind of protein called sericin, which also acts as an antimicrobial adhesive matrix to make a compact structure of macroscopic protein fibers\textsuperscript{165}. The present novel approach leads to the fabrication of a highly absorbent compact cotton patch by binding the fibers of a piece of well-stacked cotton wool with a chitosan-based soft hydrogel system which has the interesting self-healing ability (Chapter 2(A), Chapter 5). This novel technique not only exempts the spinning of cotton yarn and use of loom or other kinds of weaving machines but also makes it possible to get a mechanically robust textile material with high absorption capability. Another most interesting advantage of such a patch is that any drug, chemical or nanomaterial can be loaded in it during the time of its fabrication or after fabrication as well. The absorption capability of the hydrogel matrix coating on the cotton fiber surface facilitates the post fabrication loading of chemicals, drugs or nanomaterials. Cotton is a soft, absorbent and breathable natural fibre, making it the perfect fibre for clothing and
undergarments worn close to the skin and contains cellulose amounts to as much as 96% of the dry weight of the fibres\textsuperscript{166}.

Thus prepared hydrogel bound cotton patches were further incorporated with graphene oxide (GO), a well-known nano-filler for improvement of mechanical property along with the added advantage of having antimicrobial activity. Moreover, graphene oxide (GO), chemically exfoliated from oxidized graphite has other advantages as described in chapter 5.

At last, with the aim to come out with an effective bandage material especially for the infected wounds curcumin was introduced. Curcumin (Diferuloylmethane) is a naturally occurring phytochemical polyphenol derived from the rhizome of turmeric (Curcuma longa), a traditional medicine widely and popularly used in different parts of the world from the ancient days for treatment of several kinds of diseases including wounds and burns. Curcumin is considered to be beneficial against oxidative damage, due to its antioxidant properties and is already been proven to be helpful for better healing of the wound. There are several reports with \textit{in vitro} and \textit{in vivo} studies which demonstrate the effectiveness of curcumin in decreasing the release of inflammatory cytokines like interleukin-8 and tumor necrosis factor-\(\alpha\) from monocytes and macrophages. Moreover, it has been shown to inhibit enzymes associated with inflammation, such as cyclooxygenase and lipoxygenase. Along with the anti-inflammatory activity, curcumin has the ability to scavenge free radicals, which is the major cause of inflammation during wound healing activity. From the experimental data using animal models, recently it has been established that treatment of curcumin may assist wound healing by increasing the formation of extracellular matrix proteins and granulation tissue and neovascularization. Curcumin has the extra advantage with its proven antimicrobial activity\textsuperscript{167-172}.

\textbf{6.2. The objective:} Establishing a novel approach to fabricate compact cotton patch without weaving. Designing an effective bandage material and its trial on the animal model.

In short, this work is an endeavour to establish a method of fabrication of a soft, highly absorbent, mechanically stable compact patch from raw cotton using an interesting
biopolymeric hydrogel system having the self-healing ability. This work also studies the tuning of mechanical, structural and absorption properties of the patches introducing graphene oxide (GO) into the patches. Introduction of GO into the patches makes them antimicrobial in nature. Finally, loading of curcumin makes such patches a very effective bandage material with excellent wound healing ability which has been studied in detail.

6.3. Experimental

6.3.1. Materials used

Biopolymer Chitosan (M.W. ~ 1,93,400) was purchased from SRL, India. Absorbent cotton wool used here was obtained from ‘Perfect Surgicare’, India. Graphite nanopowder was obtained from SRL, India. Other chemicals viz., glycerol, acetic acid, sodium hydroxide, nitric acid and sulphuric acid were purchased from Merck India and used as obtained.

6.3.2. Preparation of graphene oxide

Graphene oxide was synthesized following the same procedure described in chapter 3.

6.3.3. Fabrication of compact chitosan hydrogel bound cotton patch

The biopolymer chitosan was dissolved in a 0.1 M acetic acid solution containing 20% glycerol by volume. A slice of nicely packed cotton wool of weight 1.5 g (of size ~ (6x6) cm²) was then soaked in 30 mL of that solution. The volume of the solution was adjusted in such a way, which was just the maximum volume level-the slice of cotton could absorb inside it. Then the piece of cotton wool wetted with the chitosan solution was dried in a vacuum oven at 70 °C. The dried piece of the cotton slice was then soaked in a crosslinking bath containing 1N NaOH solution for 2 hours. The chitosan hydrogel coated piece of cotton wool was then taken out from the cross-linking bath and washed for several times with distilled water. This was followed by the compression with the help of a mini lab scale compression moulding machine for 5 minutes applying a pressure of 10 tons. The compressed hydrogel bound cotton patch was then washed with distilled water and allowed
to dry again at 70 °C. Here cotton patches with 2, 5 and 20 % of chitosan with respect to the weight of the cotton were fabricated and toward this end 0.1, 0.25 and 1% (W/V) chitosan solutions were used.

6.3.4. Fabrication of compact chitosan-graphene oxide nanocomposite hydrogel bound cotton patch

Chitosan-graphene oxide nanocomposite hydrogel bound cotton patch was fabricated by following the similar procedure described for compact chitosan hydrogel bound cotton patch. First, the required amount of graphene oxide (GO) dispersion was added to a solution of chitosan dissolved in 0.1 M acetic acid solution containing 20% glycerol by volume. The required volume of that solution was then allowed to absorb by the piece of cotton wool and dried it in hot air oven at 70 °C. The dried piece of the cotton slice was then cross-linked by soaking in the 1N NaOH solution for 2 hours followed by washing and compression for 5 minutes at pressure 10 tons. The compressed chitosan-graphene oxide nanocomposite hydrogel bound cotton patch was then washed and dried again at 70 °C.

6.3.5. Loading of curcumin in the hydrogel bound cotton patch

5 mg of curcumin dissolved in minimal amount of ethanol was allowed to absorb by the cotton patches of 2 cm² diameter each. The absorption of the curcumin solution into the cotton patches was done by repeated absorption and then drying process until the whole curcumin solution get absorbed by the patch.

6.4. Characterization

The nano material and the cotton patches were characterized by Fourier transform infrared spectrometer (Nicolet 6700 FTIR) to investigate different types of interactions and functional groups present. Scanning electron microscope (SEM) images were collected on a ‘Carl Zeiss Sigma VP’ instrument. To study the mechanical properties of the fabricated compact hydrogel bound patches universal testing machine (‘Tinius Olsen 5ST’ model)
with a load cell of 2.5 KN capacity was used. The speed of the testing was maintained at 5 mm/min.

6.4.1. Antimicrobial property study

Evaluation of the antimicrobial activity of the patches was done by direct contact based method and also monitored the killing kinetics. Evaluation of the antibacterial activity was performed against *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) and *Escherichia coli* (*E. coli*) (MTCC 40).

6.4.2. Direct contact method

UV-sterilized bandage materials were subjected to antibacterial activity analysis. The test materials (sample size 1 cm²) were incubated in separate wells (24 well microtiter plate) having 2 mL of nutrient broth containing *S. aureus* and *E. coli* at concentration of 10⁷ CFU/mL. After, 48 hrs of incubation at 37 °C, 200 µL aliquots were collected and inoculated into the nutrient agar plates. After the stipulated incubation period, the culture plates were photographed for enumeration of bacterial colony forming units. To ensure validity of result, tests were conducted in triplicate.

6.4.3. Time kill assay

To determine the rate at which the fabricated bandage materials killed the tested bacteria, kill test was performed. Overnight grown cultures of the tested bacteria were washed twice in PBS (centrifugation speed 12000 xg); maintained a final concentration of approximately 2×10⁵ CFU/mL in nutrient broth and then incubated along with the bandage materials (1 cm²) at 37 °C with shaking. 0, 2, 4 and 6 hours of post treatment, samples were withdrawn and analyzed to enumerate the number of viable bacteria. The CFU of both the test bacteria were compared and plotted as the CFU/mL over time post treatment. Independent experiments were performed in triplicates.

6.4.4. In-vivo wound healing study in animals
Male Wistar rats weighing between 180-200g were procured from the animal house at Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam. All the animals were housed for a minimum of five days for acclimatization to laboratory environment before the initiation of the experiment in polypropylene cages. All the animals were fed with standard rodent pellet diet obtained from Provimi Animal Nutrition Pvt. Ltd., India and water ad libitum throughout the experimental period. The experimental room was maintained as per the standard guidelines (Temperature: 22 ± 2 °C; Relative humidity: 60–70%; 12 h–12 h light–dark cycle). All the experiments were carried during 09:00 and 17:00 h. The experimental protocol was approved (IASST/IAEC/2015-16/754) by institutional animal ethical committee (IAEC) of IASST, Guwahati which has approval from Committee for Control and Supervision of Experimentation on Animals (CPCSEA).

6.4.4.1. Wound induction: Rats were anesthetized with ketamine hydrochloride 80 mg/kg and xylazine 10 mg/kg cocktail by intraperitoneal (i.p.) injection. The dorsal region of the rats was shaved to remove the fur and sterilized the surgical area with povidone iodine solution. Further, 1.5cm×1.5cm diameter wounds were created with serial scalpel and covered with sterile gauze.

6.4.4.2. Wound infection and treatment: After 24 h of surgery, wound of the animals were infected with *Staphylococcus aureus*. Briefly-30 µL suspension of *S. aureus* containing 5 x 10^8 CFU/mL inoculated on the wounded area of mildly anesthetized (Ether) animals.

6.4.4.3. Grouping: After three days of the observation period, animals were divided randomly into five groups (n=6) for the treatment.

Group-I: Rats with wound + No infection and treated with “chitosan hydrogel coated cotton bandage”.

Group-II: Rats with wound + *S. aureus* infection and treated with “chitosan hydrogel coated cotton bandage”.

Group-III: Rats with wound + *S. aureus* infection and treated with “chitosan-GO nanocomposite hydrogel coated cotton bandage”.

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Group-IV: Rats with wound + *S. aureus* infection and treated with “chitosan hydrogel coated cotton bandage with curcumin loading”.

Group-V: Rats with wound + *S. aureus* infection and treated with “chitosan-GO nanocomposite hydrogel coated cotton bandage with curcumin loading”.

6.4.4.4. CFU count determination: Efficacy of the prepared bandage material on the *S. aureus* infected wounds was evaluated on the basis of CFU count determination on various time intervals. Briefly-on 3rd, 7th, 14th & 21st day of the treatment period, wound samples were excised and homogenized in sterile phosphate buffer saline. 100 fold tissue homogenate dilutions were plated on nutrient agar plates to enumerate the CFU count after 24 h of incubation at 37± 2 °C. Based on the excised tissue weight all the results were normalized.

6.4.4.5. Blood and tissue harvesting: Blood was collected from the animals at 7th, 14th & 21st day of post treatment through retro orbital route for the measurement of cytokine levels. At the end of the study i.e. 21st day, all the animals from each group were sacrificed and wounded area from two animals was collected in 10% buffered formaldehyde to proceed for histopathology analysis. The wounded area from remaining four animals was collected in liquid nitrogen for the biochemical estimation.

6.4.4.6. Measurement of serum inflammatory cytokine levels: Blood samples were homogenized at 1500 rpm for 10 min at 4 ºC and the supernatant was collected in separate tubes. Inflammatory cytokines like TNF-α and IL-1β levels were measured by using ELISA kits from R&D systems, USA as per the manufacturer’s instructions. All the samples were performed in the duplicate and results were expressed in Pg/ml.

6.4.4.6. Biochemical estimation-measurement of hydroxyproline: Collagen content in the wound tissue was estimated by measuring the hydroxyproline levels in the wound homogenates. Wound tissues from the different groups were dried in a hot air oven (60 ºC) and hydrolyzed in 6N hydro chloric acid for 4h at 130 ºC in air tight tubes. The pH of the hydrolysates was neutralized to 7.0 and subjected to Chloramine-T oxidation for 20 min.
The reaction was terminated by the addition of 0.4 M perchloric acid. The colour was developed by the addition of Ehrlich reagent at 60 °C and hydroxyproline content was measured spectrophotometrically at 557 nm using UV-visible spectrophotometer (Shimadzu, Japan).

6.4.4.7. Measurement of hexosamine: The wounded tissues were hydrolyzed with 6 N HCl for 8 h at 98 ºC and the resulting mixture is neutralized with 4 N NaOH, diluted with Milli-Q water. The diluted solution was heated at 96 ºC for 40 min by adding acetyl acetone solution. The reaction mixture was cooled and 96 % ethanol followed by r-dimethylaminobenzaldehyde solution (Ehrlich’s reagent) was added. Further, the resulting solution was mixed thoroughly and kept at 27 ºC for 1 h. The absorbance was measured at 530 nm by using UV-Visible spectrophotometer (Shimadzu, Japan). Hexosamine content was measured by spectral matching with the standard curve and expressed as mg/g dry tissue weight.

6.4.4.8. Histopathology of wound: Wounded tissue collected in the 10% buffered formaldehyde was preserved at least for 24 h and dehydrated gradually with ethanol from 70 to 100 %. The tissues were further cleared in the xylene and embedded in paraffin. Thin sections (4-5 µm) were prepared by using microtome and stained with hematoxylin-eosin to observe the pathological changes under the light microscope at 10x magnification.

6.5. Results and discussions

Cotton fibers were successfully coated by the first absorption of a mixture of chitosan and glycerol in a 0.1 M acetic acid medium by a piece of cotton wool followed by cross-linking of this polymeric system on the surface of the cotton fibers by neutralization with NaOH. The mechanism of formation of chitosan-glycerol hydrogel has already been explained in detail in chapter 2(A). Figure 6.1 shows a schematic presentation of the protocol followed for the fabrication of such patches. FTIR spectrum (Figure 6.2a) shows a broadening of the peak for –OH stretching in case of the coated cotton fibers compared to that of bare cotton and chitosan indicating the increase in the hydrogen bonding interaction in the coated one. The coating of the cotton fibers by the hydrogel matrix system
is vividly changed after coating. Just after the cross-linking process of the hydrogel system on the surface of the cotton fibers, the cotton wool was compressed with a compression moulding machine by applying pressure (as shown in the scheme, Figure 6.1). This compression process leads to the formation of a compact structure. After drying, this particular hydrogel system acts as an adhesive matrix due to its self-healing property and holds the cotton fibers close to one another and thus provides mechanical strength to the whole patch. Thus cotton patches with 2, 5 and 20% chitosan concentration with respect to that of the weight of the cotton fibers were fabricated. For the convenience of reporting, the patch samples were coded. Thus the chitosan hydrogel coated patch without GO is coded as CTCHx, where x represents the percentage of chitosan; patch samples with GO are coded as CTCHxGOy, where y represents the percentage of GO and x represents the percentage of chitosan. Hence, for example, patches with 2% chitosan and having GO

**Figure 6.1:** Schematic representation showing the protocol for fabrication of compact biopolymeric hydrogel coated cotton patches.
concentration 0, 0.5, 1 and 2% will have the codes as CTCH2 (patch without GO), CTCH2GO0.5, CTCH2GO1 and CTCH2GO2.

Realising the potential application of such a biopolymeric hydrogel coated patch as a smart bandage material, an endeavour was made to come out with a compact bandage material specially designed to work effectively on infected wounds. With this aim, ‘curcumin’ was chosen and loaded in the suitable patch. This is to be noted that in this work curcumin was taken as the model compound for antimicrobial agent. However, as already mentioned any antimicrobial, antifungal or antibacterial molecules can be loaded. For the use as bandage material in the animal model 5 mg of curcumin (solubilized in ethanol) was loaded in the hydrogel coated patches of diameter 2 cm$^2$ by the process of

![Figure 6.2:](image)

**Figure 6.2:** (a) FTIR spectra of cotton (CT), chitosan (CH), chitosan hydrogel coated (CTCH2) and chitosan-GO (CTCH2GO2) nanocomposite hydrogel coated patches (b) SEM images of coated and uncoated cotton fibers and different patches with varying chitosan as well as GO concentration.
repetitive absorption and drying process. The curcumin loaded patches have been coded as CTCHx+Cur (bandage without GO) or CTCHxGOy+Cur (bandage with GO).

6.5.1. Mechanical properties of the patches

The mechanical strength in the patches come because of the hydrogel coating. In their fabrication process, the chitosan hydrogel coated patches are compressed as a result of which the hydrogel coating part on the surface of cotton fibers come in contact with each other. After drying the coated fibers remains in contact with each other with the help of strong hydrogen bonding. Thus the hydrogel coating part on the cotton fibers binds the fibers with one another which eliminates the necessity of weaving and provides the patches their mechanical strength. It was observed that by varying the chitosan percentage, it is possible to tune the tensile strength, flexibility and softness of such patches. So, different

Figure 6.3: (a) Different states of the patch under stretch until break experiment in an UTM. Stress-strain profile of the cotton patches with (b) different chitosan concentration, (c) 2% chitosan and varying the GO concentration, (d, e, f) bar diagram showing comparison of tensile strength of different patches in the dry state and in the wet state after soaking in water for 24 hours.

The mechanical strength into the patches come because of the hydrogel coating. In their fabrication process, the chitosan hydrogel coated patches are compressed as a result of which the hydrogel coating part on the surface of cotton fibers come in contact with each other. After drying the coated fibers remains in contact with each other with the help of strong hydrogen bonding. Thus the hydrogel coating part on the cotton fibers binds the fibers with one another which eliminates the necessity of weaving and provides the patches their mechanical strength. It was observed that by varying the chitosan percentage, it is possible to tune the tensile strength, flexibility and softness of such patches. So, different
patches with varying chitosan concentration viz., 2%, 5% and 20% possessed an average tensile strength of about 6, 12.5 and 19 MPa respectively. Here it is noteworthy to mention that the thickness of the fabricated patches was within 0.5 to 1 mm. With the increase in the chitosan concentration, the thickness of the layer of hydrogel coating on the surface of cotton fibers increases which in turn increases the compactness of the patches as depicted by the SEM images (Figure 6.2b). Presence of higher amount of hydrogel matrix can fill the gap between the cotton fibers, connects and binds each and every fiber in a more effective way, which helps in improvement of the tensile strength of the patches. But on the other hand with the increase in the chitosan concentration flexibility of the patches decreases. After incorporation of GOs in the hydrogel matrix, the tensile strength values seemed to be improved further irrespective of the chitosan concentration present in the patches (Figure 6.3). It was also observed that 1% is the optimum concentration of GOs, which when loaded in the patches showed the maximum tensile strength value. After incorporation of 1% GO in the hydrogel matrix coating, the maximum average tensile strength values reached to ~10, 19 and 27.5 MPa for the patches with 2, 5 and 20% chitosan respectively. GO with its aromatic structure acts as an effective filler for improvement of mechanical property in polymeric nanocomposite systems. Moreover, the presence of polar groups on the surface of GO facilitates good compatibility with the chitosan hydrogel matrix and increases the crosslinking density of the hydrogel matrix system which as a whole enhances the compactness of the patches. But at higher concentration of GOs, the tensile strength value decreases due to the overloading of GO nanomaterials. The nature of the stress strain curve clearly shows the tough nature of the patches. Since the patches are totally fibrous inside, so they do not possess any sharp breaking point. So the stress-strain profile of the patches was collected up to a minimum force of 15N for each sample. This fibrous system is actually responsible for the tough and flexible nature of such patches as a result of which they can be easily bent like any elastic material as per application requirement. The mechanical strength of the patches is seemed to be comparable or even better compared to some already reported polymeric systems for bandage application. But such patches have the advantages over polymeric systems because most of the bio-polymeric materials lose their mechanical strength when they come in contact with water.
because of their solubility in aqueous medium. Retention of mechanical strength of such patches in presence of water was also studied and this revealed that almost all the patches retained above 60% of their original strength in the wet condition and even after dipping into the water for 24 hours. This retention of mechanical strength is because of the use of a water insoluble hydrogel matrix system for binding the cotton fibers.

6.5.2. Evaluation of antimicrobial activity of the patches

Cotton based patches with GO and curcumin exhibited significant antibacterial activity against both pathogenic gram positive and gram negative bacteria, viz., *Staphylococcus aureus* and *Escherichia coli*. The detail antimicrobial activity evaluation of the patches was carried out by two different methods. In the first method, after incubating the bandage materials with $10^7$ CFU of test bacteria, the aliquots from the respective culture tubes were cultivated in nutrient agar plates to tally the number of bacteria seeded onto the patches were $10^7$ CFU/mL (b) CFU count of *E. coli* and *S. aureus* at different intervals on treatment with different patch samples.

**Figure 6.4:** (a) Typical photographs of re-cultivated microorganism colonies for *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) on nutrient agar culture plates, where the concentrations of bacteria seeded onto the patches were $10^7$ CFU/mL (b) CFU count of *E. coli* and *S. aureus* at different intervals on treatment with different patch samples.
viable bacterial cell count. Figure 6.4(a) represents the typical photographs of re-cultivated $(10^7 \text{ CFU/mL})$ $E. \text{ coli}$ and $S. \text{ aureus}$ colonies after treatment against four types of patches (viz. CTCH2, CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur). Both CTCH2GO2 and CTCH2+Cur patches were seemed to exhibit antimicrobial activity by reducing the bacterial colonies on the re-cultivated agar plates after treatment. The study of the time killing assay also shows the reduction of the CFU count with time for both the pathogenic microorganism (Figure 6.4(b)). The antimicrobial activity of GO and its mode of action has already been well established by several research groups (Chapter 5). Upon direct contact with the microbial cell, the sharp edges of GO nano-sheet result in membrane stress and subsequent superoxide anion independent oxidation, which in turn, led to the oxidation of proteins, lipids and nucleic acids and ultimately resulted in the membrane damage and cell death. On the other-hand curcumin is also a well-known antimicrobial agent and as per experimental report it can induce membrane permeabilization of the bacterial cell and leads to membrane damage and leakage whichintern kill the bacteria$^{177}$. CTCH2GO2+Cur treatment resulted in the absence or minimal growth of microorganism colonies; which depicts significant antibacterial activity of the same due to the combined action of both the antimicrobial agents.

6.5.3. Evaluation of biocompatibility and cytotoxicity of the patches

Biocompatibility and cytotoxicity of the patches that are going to be used as wound dressing material was also studied. This was done by two different methods, viz. haemolytic activity study and MTT assay study.

6.5.3.1. Haemolytic activity study: In biosafety evaluation studies of various biomedical materials, human RBCs have been widely used which constitutes 40-50% (v/v) of human whole blood. In this study, effect of the prepared bandage materials on the lysis and morphology of the RBCs was evaluated. Upon treatment for 1 h, it was found that the tested bandage materials (CTCH2, CTCH2GO2, CTCH2+Cur & CTCH2GO2+Cur) did not induced significant hemolysis. The hemolysis percentage of all the tested materials is far below the permeable limit (5%)$^{159}$ which suggests that the bandage materials are
hemocompatible (Figure 6.5(b)). Particulate state, particle size, surface charge/oxygen content and the extent of exfoliation of GO play a crucial role in hemolysis. Smaller the particles the greater are the hemolysis, whereas GO sheets in aggregated form demonstrate mild hemolysis. In this present study, electrostatically adsorbed chitosan covers the GO sheets and abolish the hemolytic potential\(^{158}\). To confirm further, RBCs after treatment with bandage materials were subjected to FE-SEM analysis. Naturally, the shape of mature RBCs is like biconcave disk and they are very sensitive to interactions with membrane-active substances. Interaction with CTCH2 & CTCH2GO2 did not distort the morphology of RBCs, whereas CTCH2+Cur & CTCH2GO2+Cur treatments resulted in a slight alteration in shape (Figure 6.5(a)). These alterations are due to the presence of curcumin which is a well-known anti-cancer agent.

6.5.3.2. MTT assay: MTT assay was also performed to explore the cytotoxicity of the prepared bandage materials. CTCH2 and CTCH2GO2 exhibited negligible cytotoxic effects on the mitochondrial activity of mouse L929 fibroblastic cell line. CTCH2+Cur & CTCH2GO2+Cur demonstrated mild effects on the cell viability compared to that of the former two, which might be due to the presence

\[\text{Figure 6.5: (a) Scanning electron microscopy (SEM) of human blood cells incubating with distilled water (DW), saline water (SW) and different bandage materials viz.; CTCH2, CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur (b) Percent hemolysis of human blood cell by different types of bandage materials (c) MTT assay study result.}\]
of curcumin in both samples (Figure 6.5(c)). More than 80% cell viability (~ 92%, 86%, 84% and 82% for CTCH2, CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur respectively) was found for all the samples after treatment for 72 hours. There are only a few studies could be found on investigations of in vitro cytotoxicity of GO materials. GO is reported to have negligible toxicity towards human alveolar epithelial A547 cells, A549 cells and mouse fibroblast L929 cells\textsuperscript{178}. It is also reported that GO substrates are highly biocompatible and possess enhanced gene transfection efficacy in NIH-3T3 fibroblast\textsuperscript{179}. Though GO is reported as an antimicrobial nano material it does not induces mammalian cell toxicity. Size, surface charge, surface functional groups, residual precursors and particulate state are the possible parameters to be considered in regard to GO cytotoxicity. Curcumin, one of the components of the prepared bandage materials is a well-known chemotherapeutic anticancer agent and possesses significant wound healing property. It was reported that unlike other anti-cancer agent curcumin does not induce any toxicity towards normal cells\textsuperscript{31}. In this present study, mouse fibroblast cells were found to be non-sensitive to CTCH2+Cur and CTCH2GO2+Cur as concluded from MTT results. The minimal cell death shown by CTCH2+Cur and CTCH2GO2+Cur is may be due to disturbance created by the bandage materials in the physiological environment of incubated cells.

6.5.4. Absorption properties of the patches

The patches were found to possess excellent absorption capability when a time dependent absorption study in water was carried out. The swelling ratios of the patches were calculated with the help of the formula given below\textsuperscript{86}.

\[
\text{Swelling ratio (\%) } = \left[ \frac{(W_s - W_d)}{W_d} \right] \times 100\%
\]

where, Ws and Wd are the weights of the swollen and dried samples, respectively. The time dependent swelling ratio of the samples are shown in Figure 6.6. The absorption property of the patches is also influenced by the amount of chitosan present in the patches. The patch CTCH2 (i.e. the patch with 2% chitosan) shows the maximum water absorption ability with more than 200% of its original weight (S.R \(\sim\)236%) within 5 sec and after 2
hours, it reaches a saturation absorption with S.R. ~280%. The patch CTCH5 and CTCH20 absorbs ~180% and 147% within 5 sec with saturation absorption of 195% and 193% after 2 hours respectively. The high absorption capability of the patches is mainly because their

**Figure 6.6:** Graphical representation of the comparative study of absorption properties of different patches in water with varying chitosan and GO concentration (a, b, c, d) and after loading of curcumin (e). Absorption properties of different patches with 2% chitosan concentration and varying GO concentration in blood plasma (f).
porous structure, although the hydrogel matrix coating part also has the capability to absorb water to some extent. That is why the patches with less chitosan content shows higher absorption capacity because of their less compactness compared to the higher chitosan containing patches which is vividly perceptible from the SEM images (Figure 6.2(b)). Although the absorption property of the patches can be understood from the measurement of swelling ratio (Figure 6.6), but to provide a direct quantitative idea, it is mentioned here that a small patch of size (4 × 4 cm²) and thickness ~1mm can easily absorb ~2 mL (by CTCH2 and CTCH2+Cur) or ~1.7 mL of water (by CTCH2GO2 and CTCH2GO2+Cur) instantly. This absorption capability is again directly proportional to the volume of the patches. Thus the absorption capability of the patches can easily be increased by increasing their thickness, without changing the area. The thickness of the patches can be increased just by increasing the thickness of the cotton wool used for their fabrication. As the compactness of the patches increases after incorporation of GO, hence the absorption capability of the GO incorporated patches decreased to some extent, but still, they possess very good absorption capability. To test the effectiveness of the patches as bandage material it was attempted to mimic the real environment of the wound and the absorption of the patches were tested against blood plasma. The patches showed very satisfactory absorptivity against blood plasma (Figure 6.6f), although the SR value is only just a little less than water which may be due to the presence of high protein content in blood plasma increasing the viscosity of the whole liquid substance.

6.5.5. Wound-healing ability of the patches

An in-vivo wound healing study for a period of 21 days was carried out using four different types of bandage materials, viz., CTCH2, CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur on S. aureus infected excised wounds in Wistar rats. Among the cotton patches, the patches with chitosan concentration 2% were preferred for wound dressing application and test in the animal model because of their sufficient flexibility, softness and higher absorption rate. Photographic images of the wounds treated with different bandage materials and their healing with time within the period of observation are shown in Figure 6.7. A non-infected wound covered with CTCH2 bandage patch is also shown by the
images. Interestingly, the infected wounds treated with the CTCH2GO2+Cur bandage patch showed faster and satisfactory wound healing rate along with the hair growth in the wounded area within the days of observation. This effective wound healing ability of such bandage patches come from GO, a highly active antimicrobial agent and curcumin, the active part of the most popular drug of Ayurveda for wound treatment i.e. ‘Turmeric’. Antimicrobial activity of curcumin along with its faster and effective wound healing ability has already been well established.171,172 The combined effect of GO and curcumin makes the CTCH2GO2+Cur bandage patch an excellent smart wound dressing material for the wounds infected by microbes. Along with fastening the wound healing, such bandage materials also take care of the pus material ejaculated from the degraded wounds with microbial infection by their property of high absorptivity. In addition, such patches with their porous nature (as evident from the SEM images) provide ventilation to the wound which is one of the significant advantages of such patches. It is noteworthy to mention here that although few reports have been found on wound healing ability of curcumin and

Figure 6.7: (a) Observation of post-operative wound healing of animal wounds treated with different bandage treatments at various time intervals (b) microscopic images showing the histopathology of the wounds after completion of the period of observation.
graphene based bandage materials, a thorough literature search indicates that this is the first report to study the ability of wound healing by a bandage material with curcumin, graphene oxide and the combination of the two on an infected wound.

To monitor the healing of the wounds treated with bandages, loaded with different agents, various aspects which can indicate the curing of the wounded area were studied.

6.5.5.1. Effect on S. aureus load: S. aureus load on the infected excised wounds were measured in terms of CFU count in respective post-operative days (3rd, 7th, 14th & 21st day). Both CTCH2GO2 & CTCH2+Cur treated group of wounds demonstrated the significant decrease in the S. aureus CFU burden compared to the CTCH2 treated wounds. Both CTCH2GO2 and CTCH2+Cur treated wounds showed almost equal results while in case of the CTCH2GO2+Cur treated wounds S. aureus concentration decreased significantly (Figure 6.8).

6.5.5.2. Effect on connective tissue markers: Collagen being a major component of connective tissue provides a frame work for structural strength and helps in tissue regeneration. Augmented levels of hydroxyproline and hexosamine are regarded as one of the crucial parameters for the wound healing process as the increase in both the markers is an index of neo collagen synthesis.

![Graphs](attachment:Graph6.8.png)

**Figure 6.8:** Colony forming unit (of infected S. aureus) data from the excised wound treated with different bandage treatments at various time intervals (a). Effect of surgery and different bandage treatments on (b) plasma pro inflammatory cytokine Tumor necrosis factor α levels and (c) plasma pro inflammatory cytokine Interleukin 1β levels and at different time intervals.
Chapter 6

Treatment with CTCH2GO2 & CTCH2+Cur bandage materials resulted in significant increase in both hydroxyproline and hexosamine content than CTCH2 treated infected wound areas at different time intervals (7th, 14th & 21st day). Whereas, CTCH2GO2+Cur treatment resulted in significant acceleration in both the marker’s level as compared to remaining treatment groups (Table 6.1). Repair and healing of injured tissues involves hemostasis, inflammation, proliferation and remodeling. Microorganisms, sequestered at the skin surface gain access to the underlying tissues through injured skin which leads to delayed wound healing. Wound healing follows a cascade of complex processes for eradication of invaded microbial pathogens from the effected tissue which ultimately leads to remodeling of the same. GO containing bandage materials significantly ameliorate wound healing process through antibacterial action. Curcumin is well known for its wound healing and antimicrobial potential. Curcumin improves healing of injured tissues through modulating collagen synthesis and decrease in ROS generation\textsuperscript{181}.

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Treatments</th>
<th>Hexosamine (mg/100mg of tissue)</th>
<th>Hydroxyproline (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7\textsuperscript{th} day</td>
<td>14\textsuperscript{th} day</td>
</tr>
<tr>
<td>1</td>
<td>CTCH2 @Normal wound</td>
<td>0.52 ± 0.06</td>
<td>0.69 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>CTCH2 @Infected wound</td>
<td>0.12 ± 0.04</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>CTCH2GO2 @Infected wound</td>
<td>0.32 ± 0.08</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>4</td>
<td>CTCH2+Cur @Infected wound</td>
<td>0.39 ± 0.1</td>
<td>0.61 ± 0.11</td>
</tr>
<tr>
<td>5</td>
<td>CTCH2GO2+Cur @Infected wound</td>
<td>0.58 ± 0.07</td>
<td>0.77± 0.08</td>
</tr>
</tbody>
</table>

Table 6.1: Hexosamine and hydroxyproline content of granulation on different days of healing after treating the wound with different bandage treatments.

6.5.5.3. Effect on inflammatory cytokine levels: In this present study, CTCH2 treated infected wounds showed higher levels of both inflammatory cytokine (IL-1β & TNF-α). CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur treatment significantly decreased both the cytokines where, CTCH2GO2+Cur showed better results among all treatments. TNF-α and IL-1β alters the matrix metalloproteinase synthesis resulted in increased collagenase activity\textsuperscript{182}. Infections resulted pronounced inflammatory response which is governed by an
elevated production and release of inflammatory cytokines. GO reduced the bacterial load at the wound site which leads to decrease in the levels of inflammatory markers and fasten the wound healing process. Curcumin has also been found to inhibit the release of proinflammatory cytokine (IL-1β & TNF-α) from monocytes and macrophages. It is also reported that curcumin possesses the ability to inhibit the inflammatory enzymes such as cyclooxygenase and lipoxygenase levels and helps in fasten the wound healing process.

6.5.5.4. Histopathology of wounds: Histopathology analysis of the wounded area was performed after 21 days post treatment with different bandages to confirm the wound healing process. In the sample collected from CTCH2 treated infected wounds, dead cells with chronic cell infiltrate was observed indicating no sign of healing. Treatment with CTCH2GO2, CTCH2+Cur and CTCH2GO2+Cur where the apparent wound healing was visible also showed the neovascularization, epithelialization, collagenase tissue with all the adnexal structures like sweat glands and hair follicles. Complete wound healing was observed for the infected wound on treatment with CTCH2GO2+Cur bandage along with the normal wound with almost nil inflammatory cell infiltrate.

6.6. Conclusion

To summarise, a type of biopolymeric hydrogel bound cotton based compact patches were fabricated by a compression technique. The structural design of such patches allows to load different types of chemicals, drugs, nano or micro structured materials to modify their properties. Mechanical properties of the patches could be tuned by varying the percentage of the biopolymer used. Such patches retain their mechanical strength even in the wet state and soaking in water for 24 hours. Incorporation of GO not only provided the antimicrobial activity but it also improved the mechanical strength of the patches. After loading of curcumin, the antimicrobial activity of the GO incorporated patches further enhanced. All the patches possess good absorption capability. Patches are non-cytotoxic in nature as proven from the hemolysis and MTT assay test. Use of this patches as bandage material in animal model depicted the efficiency of such patches as the wound dressing.
material. The loading of curcumin in the GO incorporated patches showed the maximum wound healing rate compared to the other bandage patches.