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Antoniyar Arockiaraj (antoniyarbiochem@gmail.com)  
Anna University, Chennai

Subramanian Sundaramoorthy  
Anna University, Chennai

Research Article

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Synthesis and characterization of amine functionalized cellulose nanogel for transdermal drug delivery

Antoniyar Arockia raj*, Subramanian Sundaramoorthy
Department of Textile Technology, Anna University, Chennai 600025, India
Corresponding author email: antoniyarbiochem@gmail.com

Highlights

- AFCNC was synthesized by acid hydrolysis, TEMPO oxidation, and amidation
- Biodegradable AFCNG was prepared through regeneration chemistry
- AFCNG has minimal hemolytic activity and low cytotoxicity to 3T3 cells
- Curcumin-loaded AFCNG shows sustained release of drug and cytotoxicity to B16-F10 cells
- Curcumin-loaded AFCNG has the potential for skin cancer treatment
Graphical Abstract
Fig. 1 Schematic illustration of preparation of AFCNG form AFCNC
Fig. 2 Schematic diagram of the preparation of curcumin loaded AFCNG.
Abstract

This study presents the synthesis and characterization of an amine-functionalized cellulose nanogel (AFCNG) for potential biomedical applications. AFCNG was synthesized using a combination of acid hydrolysis, TEMPO oxidation, and EDC/NHS-mediated coupling via amide linkage. Biodegradable AFCNG was formulated with a mean size of 74 nm through a regeneration process. Characterization of AFCNG was performed using various techniques such as FTIR spectroscopy, XRD, XPS, Zeta potential, DLS, HRSEM and TGA. The results revealed that AFCNG was amorphous, highly stable in colloidal form, and had higher degradation temperature and water absorption capacity. Biodegradation of AFCNG was also assessed by incubating it with lysozyme for five weeks, which resulted in a degradation level of 83%. Furthermore, hemo-compatibility and cytotoxicity properties of AFCNG were evaluated, and the results showed that it was hemo-compatible and non-toxic to 3T3 cells by cellular morphology assessment with DAPI staining. Curcumin-loaded AFCNG exhibited high cytotoxicity to B16-F10 cells, suggesting its potential as a transdermal drug delivery system for skin cancer treatment. The study concluded that AFCNG possesses the necessary characteristics to serve as a carrier for transdermal drug delivery applications.

Keywords: Amine-functionalized, Biodegradable polymer, Cellulose nanogel, Curcumin, Cytotoxicity, Transdermal drug delivery

List of symbols and abbreviations

AFC - Amine functionalized cellulose

AFCNC - Amine functionalized cellulose nanocrystalline

AFCNG - Amine functionalized cellulose nanogel

CAFCNG - Curcumin loaded Amine Functionalized Cellulose Nano Gel
CNC - Cellulose nanocrystalline

FR - Folate receptors

MCC - Micro crystalline cellulose

NCC - Nano crystalline cellulose

OCNC - Oxidized cellulose nanocrystalline

TEMPO - (2,2,6,6-tetramethylpiperidine-1-oxyl)

Introduction

Transdermal drug delivery is an attractive approach for drug administration due to its non-invasive nature, self-administration, lower daily dose, direct access to the target site, and avoidance of first-pass metabolism [1]. In comparison to injections, transdermal delivery also eliminates the risk of disease transmission through needle reuse and reduces medical waste [2–4]. There are three generations of transdermal drug delivery systems: the first generation comprises transdermal patches for small lipophilic drugs, the second generation involves chemical enhancers that produce skin irritation, and the third generation includes physical enhancers that cause a burning sensation on the skin [5]. The skin's stratum corneum, a continuous lipid bilayer, usually prevents drug permeation, making it challenging to develop transdermal drug delivery systems [6, 7]. Despite significant advances in enhancing skin permeability using physical and chemical methods such as micro needles [8], chemical/lipid enhancers [9], laser ablation [10], iontophoresis using electric fields [11], electroporation [12], and pressure waves generated by ultrasound or photoacoustic effects [13], these approaches have drawbacks [14, 15].

Nanotechnology has emerged as a prominent technology in various fields [16], including biomedicine, to address the challenges associated with conventional drug delivery systems. Among the various nanosized particles, bio-polymeric nanogels have gained
attention due to their unique characteristics [17]. These nanogels are cross-linked hydrophilic polymeric networks, which can be linked physically or chemically, making them a promising candidate for drug delivery applications [18]. The nanogels have a high content of functional groups such as amide, carboxylic, and hydroxyl, which enable them to exhibit high water uptake, a large surface area for bioconjugation, a porous structure for high drug loading, and controlled drug release. Furthermore, these nanogels are stable in aqueous media and biological fluids, making them attractive for drug delivery applications. They respond rapidly to external stimuli in specific chemical and biological environments, which adds to their appeal for drug delivery [19].

Cellulose is a highly sought-after material for biomedical applications due to its unique characteristics such as abundance, renewability, biocompatibility, biodegradability, low cost, and low toxicity [20]. One of the basic building blocks of cellulosic materials, cellulose nanocrystals (CNC), can be extracted by acid hydrolysis [21, 22]. CNC offers remarkable properties such as low density, low coefficients of thermal expansion, high surface area, and optical and electrical functionality [23]. Glyco-polysaccharides such as CNC are a green and attractive alternative to other functional nanoparticles such as fullerenes and carbon nanotubes, which are not biodegradable [24]. CNC can be further oxidized by TEMPO oxidation and used in biomedical applications [25–27], especially in drug delivery [28] and wound healing applications [29]. By amidation with amines, TEMPO-oxidized CNC can produce cellulose amide derivatives with poly(ethylene glycol) [30] and amino acid/peptide moieties [31]. These amide groups have the potential to be effectively used in drug delivery applications and address the aforementioned problems [32].

In this connection, folic acid (FA) is an ideal targeting agent due to its high receptor affinity, non-immunogenicity, low molecular weight, stability, cost-effectiveness [33], and ease of chemical conjugation compared to other target proteins[7]. Folate receptors (FR) are
over-expressed on various malignant tumors, including skin cancer, but are limited in healthy tissues and organs [7, 34, 35]. Moreover, β-alanine is an essential molecule for drug delivery applications, as it is biocompatible, biodegradable, and non-toxic [36]. In this respect, the surface modification of cellulose was combined with primary amino group of folic acid to form amide group [37] which has enhanced the binding ability [32, 38, 39]. At this junction, the OCNC was joined with folic acid conjugated β-alanine moieties, followed by preparation of a nanogel through regeneration chemistry [40] which makes it a promising candidate for skin penetration in drug delivery applications. The nanogel has good biocompatibility, biodegradability, and low toxicity of the functional group of hydroxyl, carboxyl and amide, making it an excellent carrier for drug delivery applications. The amine functionalized nanogel has effectively improved hydrophilic, biocompatible, biodegradable, and highly beneficial for drug delivery applications. However, the preparation of AFCNG formulation using regeneration chemistry has not been reported yet. In the view of above characteristic feature, curcumin loaded AFCNG formulation can be used to improve physicochemical property [2, 6, 7] via the receptor mediated endocytosis.

The aim of the present study was to synthesize AFCNC and AFCNG using various techniques such as acid hydrolysis, Tempo oxidation and EDC/NHS-mediated coupling. The synthesized MCC, CNC, OCNC, AFCNC and AFCNG were characterized for their surface morphology, functional groups, swelling, thermal and structural properties. Additionally, the cytotoxicity, hemolysis, biodegradation, and cell assessment were conducted to evaluate the biocompatibility of these materials. The morphology of the 3T3 cells was also examined using DAPI staining. Furthermore, in vitro drug release studies were conducted to evaluate the efficiency of the AFCNG system for drug delivery. Finally, the cytotoxic effect of the anionic curcumin loaded AFCNG was evaluated against B16-F10 cells expressing folic acid receptors via receptor-mediated endocytosis.
Materials and Methods

Materials

The following chemicals were used in the study: microcrystalline cellulose MCC (with a particle size of 51 µm, CAS Number: 9004-34-6), folic acid (CAS no: 59-30-3, molecular weight: 441.40), β-alanine (CAS No: 107-95-9, molecular weight: 89.09), 12 kDa MW cut-off dialysis membrane, 4,6-diamidino-2-phenylindole (DAPI) (CAS No: 28718-90-3, molecular weight: 350.25), N-hydroxysuccinimide (NHS) (CAS No: 6066-82-6, molecular weight: 115.09), sodium bromide (NaBr) (CAS No: 7789-38-0, molecular weight: 150.89), morpholinoethanesulfonic acid (MES) (CAS No.: 4432-31-9, molecular weight: 195.24), curcumin (CAS no: 458-37-7, molecular weight: 368.38), (3-dimethylaminopropyl)-N-ethylcarbodimmide hydrochloride (EDC) (CAS No: 25952-53-8, molecular weight: 191.70), sodium hydroxide (NaOH) (CAS No: 1310-73-2, molecular weight: 40.00), sodium chloride (NaCl) (CAS No: 7647-14-5, molecular weight: 58.44), and calcium chloride (CaCl2) (CAS No: 10035-04-8, molecular weight: 147.02) were purchased from Sigma Aldrich. TEMPO (CAS No: 2564-83-2, molecular weight: 156.25), cellulose acetate dialysis membrane (LA390, average flat width 25.27 mm, average diameter 15.9 mm and capacity approx. 1.99 ml/cm), hydrochloric acid, sulphuric acid, methanol and 13% sodium hypochlorite were used. All the chemicals used in the study were of analytical reagent (AR) grade and were used without further purification. Double-distilled (DD) water was used for all experimental methods.
Isolation of CNC

The scheme showing preparation of AFCNG is given in Figure 1. CNC was synthesized using acid hydrolysis method prescribed by Wen bai [22] and Lee [21]. MCC was initially added to deionized water. The suspension was kept in ice bath cooling and stirred with a magnetic stirrer. The concentrated sulphuric acid (64 %) was added dropwise into the suspension. Then, it was stirred at 45°C for an hour. The resulting suspension was quenched in cold deionized water to terminate the hydrolysis reaction. Once the suspended cellulose was precipitated, the turbid was discarded and replaced with fresh deionized water. The suspension was centrifuged with deionized water at 12,000 rpm for 10 min. The centrifugation cycle was repeated until the supernatant became clear. The suspension was dialyzed with deionized water for 7 days in a dialysis membrane (12 kDa MW cut-off). The resulting suspension was sonicated for 30 min then spray-dried to get CNC powders.

Preparation of TEMPO oxidized CNC

TEMPO-mediated oxidation has been carried out earlier by Hemraz et al [37] and Lin [26]. In this work, the procedure prescribed by Lin [26] is followed. A 0.5 gram of spray dried CNC was added with 50 mL of distilled water. TEMPO (14.75 mg) and NaBr (162 mg) were dissolved in 50 mL distilled water and the solution was added slowly into the CNC suspension. The suspension was added with 8.1 mL NaOCl (13 %) solution to start the oxidizing reaction. The suspension was stirred for 3h at room temperature at pH 10 by adding 0.5 M NaOH. Acidic carboxyl groups were formed by the oxidation reaction. The reaction was terminated by the addition 4 mL of methanol and the pH was adjusted to 7 with 0.5 M HCl. The suspension of oxidized cellulose nanocrystals(OCNC) was centrifuged thoroughly with water for three times and dialyzed using membrane (12 kDa MW cut-off) against deionized water and freeze dried.
Preparation of AFCNC

FA-β-alanine was synthesized by EDC/NHS-mediated coupling via amide linkage as reported by Hashemkhani et al [39]. Folic acid weighing 3.307 g was mixed in 100 mL of MES buffer (0.1 M, pH 6.5) and stimulated for 30 min with 1.4377 g EDC and 0.863 g NHS. The reaction solution was then mixed with β-alanine 0.668 g and allowed to stand overnight at room temperature in the dark. FA-β-alanine moieties was conjugated and the mixture was centrifuged with demonized water dialyzed (12kDa MW cut-off) for 1 day and then freeze dried.

Aminated CNC was synthesized by EDC/NHS-mediated coupling via amide linkage reported previously by Hemraz et al [37]. The oxidized CNC (340 mg) was sonicated with 2-(N-morpholino)-ethanesulfonic acid buffer (20 mL) at pH 5 for 20 minutes. The suspension of oxidized CNC was added into EDC (602 mg), NHS (361 mg) and stirred for 1 h. FA-β-alanine moieties (2.720 g) was then added and the mixture was stirred for 24 h at room temperature. The mixture was dialyzed using 12 kDa MW cut-off membrane for 1 day against saturated sodium chloride solution and then against distilled water until no free amines was identified in the dialysate. The AFCNC was then freeze dried.

Preparation of AFC nanogel

The AFC nanogel was prepared using controlled regeneration chemistry approach of Reginold and Tamura [38, 40]. Five grams of AFCNC was added to 1 L of saturated CaCl₂ solution in methanol (85 %), which was vigorously agitated using magnetic stirrer for 48h at room temp. The solution was filtered to remove the undissolved material. To prepare homogeneous AFCNG, the solution was stirred for 1 hour using a magnetic stirrer and the methanol was added to the solution in drop-by-drop while stirring constantly. The nanogel was regenerated. Thenanogelturbid solution was probe sonicated for 15 min and then centrifuged. The suspended AFCNG was washed with water and stored.
Analytical characterization

Attenuated Total Internal Reflectance (ATR-IR) spectra of MCC, CNC, OCNC, AFCNC and AFCNG were taken using BRUKER-ALPHA-Platinum-ATR-IR spectrometer for 100 scans per sample in transmittance mode at spectral range of 500 to 4000 cm\(^{-1}\), resolution of 2 cm\(^{-1}\) to analyze functional groups of compounds. X-ray photoelectron spectroscopy (XPS) spectra were taken using XPS spectroscope (Physical Electronics, PHI 5000 VersaProbe III, US). The peaks were analyzed using software for structural analysis.

Crystalline property of materials was studied using BRUKER D8 ADVANCE POWDER X-Ray Diffractometer with high speed wide angle lynx eye detector. The crystal structure of MCC, CNC, OCNC, AFCNC and AFCNG was determined by using Seigal method. The relative amount of crystalline material in the sample expressed as the Crystallinity Index (CrI) was calculated using Equation (1).

\[
\text{CrI} = \left( \frac{I_{cr} - I_{am}}{I_{cr}} \right) \times 100
\]

Where \(I_{cr}\) the intensity of crystalline peak and intensity of amorphous \(I_{am}\) is the intensity corresponding to amorphous peak.

As the biomaterials are sterilized using higher temperatures, thermogravimetric analysis was carried out to understand the thermal stability of samples. Thermogravimetric study was carried out using with NETZSCH STA 449 F3 under \(\text{N}_2\) atmosphere at a heating rate and cooling rate of 10 °C/min from atmospheric temperature to 600 °C. High Resolution Scanning Electron Microscope (HRSEM, FEI-Quanta FEG 200F) was used to study surface morphology of samples. Particle size distribution and zeta potential measurement were performed using HORIBA SZ-100 at 27°C. The drug loading and releasing of samples were studied using UV-visible spectroscopy (Perkin Elmer LAMBDA 950, UV-VIS-NIR Spectrophotometer). The drug conjugation of samples was investigated using Fluorescence Spectroscopy (Jobin Yvon Fluorimeter, Spectrofluorimeter).
Water absorption

The water absorption study of AFCNG was investigated at three pH levels viz., 4, 7 and 9. The dry weight (Wo) of nanogel was measured, then it was placed in the buffer solution of specific pH for 5 min at room temperature. Three different pellets of AFCNG were used for each of this experiment. Filter paper was used to remove excess unattached water content from the pellet, and then the wet weight (Ww) was measured. The absorption % was calculated using the Equation 2.

\[
\text{Absorption} \% = \frac{W_w - W_o}{W_o} \times 100
\]

Eq. (2)

Biodegradation

It is expected that the carrier AFCNG loaded with drug should degrade by lysosome and release the drug to the nucleus. Hence, degradation study was carried out with lysozyme hydrochloride solution (1.5μg/mL) at 37°C. The lyophilized pellets of dry weight (Wo) were immersed in lysozyme hydrochloride solution for forty two days. The solution was changed every 24h to keep it active enzymatically. After keeping AFCNG for seven, fourteen, twenty one, and twenty eight and thirtyfive days in the enzymatic media, they were cleaned with distilled water before being freeze dried. The treated dry samples were then weighed (Wt). The biodegradation was observed by tracking the weight reduction over time. The degradation % was calculated using the Equation 3.

\[
\text{Degradation ratio} \% = \frac{W_o - W_t}{W_o} \times 100
\]

Eq (3)

The degradation was also observed using SEM images.
Hemolysis

Blood compatibility of AFCNG was examined using a hemolytic assay. Human blood collected from a healthy volunteer in a 3.8 % sodium citrate coated tube was diluted with phosphate buffer saline (PBS, pH 7.4) in the ratio of 1:20 (v/v). The blood diluted with PBS was taken as a negative control, and the blood with Triton X was taken as a positive control. The AFCNG was autoclaved. The AFCNG of different conc. was prepared by dissolving it in DMSO in the range from 0.4-2 mg/ml in steps of 0.4 mg/ml. The sample of 100 µL was added to 900 µL of blood and incubated for 60 min at 37°C. Then, the samples were spun at 3000 rpm for 10 min and the supernatant was collected. The optical density value (OD) of the supernatant was measured at 545 nm using spectrophotometer and the hemolytic rate was calculated using Equation 4.

\[
\text{Hemolysis} = \frac{\text{OD Sample} - \text{OD Negative control}}{\text{OD positive control} - \text{OD Negative control}} \times 100
\]

Cytotoxicity study

MTT assay was used to evaluate the cytotoxicity of the AFCNG against 3T3. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide) assay is a colorimetric test based on the selective ability of live cells to reduce the tetrazolium component of MTT into purple-coloured formazan crystals. 3T3 (mouse embryonic fibroblast cell line), was cultured (100 µL) in 96-well plates at a density of 1×10^5 cells per well for 24h at 27°C and 5% CO₂. After confluence, the AFCNGs of conc. 0.2, 0.4, 0.6, 0.8, and 1 mg/ mL were placed on the cells of well plate. The well plate without AFCNG is considered as control. After the incubation time of 24h, 5 µL of MTT reagent (10 mg/mL) was added to the medium and incubated for 4h at 37°C, 95% RH in an incubator containing 5%CO₂. Subsequently, the medium was discarded and 200 µL of dimethyl sulfoxide (DMSO) was added to record its optical density using spectrophotometer at 540 nm. The optical density value (OD) has varied
linearly with the viable cell population. The cell viability percentage was calculated using Equation 5.

\[
\text{Cell Viability} \% = \frac{OD_{\text{test sample}}}{OD_{\text{control}}} \times 100 \quad \text{Eq. (5)}
\]

**Cellular assessment by DAPI staining**

The 3T3 cells were placed in 24-well plates. The cells were then incubated for 24h to allow the cells to adhere to the well. After 24h of incubation, the media was changed, and the wells were gently rinsed with PBS. AFCNG of conc. 1 mg/ml was then added to the wells along with the media in triplicate and incubated for 24h. The cells were rinsed three times with PBS, fixed with 5% para-formaldehyde for 30 min. and stained for 10 min. with 1 µg of PBS-DAPI. The cell without the addition of AFCNG is considered as control. The cells were then visualized under a fluorescent Imaging System, BioRad, USA.

**Curcumin loading, Release and Cytotoxicity study**

The scheme representing preparation of curcumin loaded AFCNG is given in Figure 2. A 5mg of curcumin was dissolved in 5 ml of methanol and this solvent was added to 5 ml of AFCNG (25 mg of AFCNC). This solution was stirred for 12 h to homogeneously mix curcumin in the AFCNG. The solution was centrifuged to remove excess unbound drug. Then it was added dropwise to methanol to regenerate curcumin and entrap with AFCNG by forming yellow colored solution. This mixture was centrifuged with water and sonicated to bring down the prepared gel to nano regime. The drug loading was calculated using Equation 6.

\[
\text{Drug loading} \% = \frac{\text{Weight of drug in nanocomposite}}{\text{Weight of nanocomposite}} \times 100 \quad \text{Eq. (6)}
\]

The drug entrapment efficiency was estimated using UV vis spectrophotometer at 420 nm.

\[
\text{Drug entrapment efficiency} \% = \frac{OD \text{ value of AFCNG loaded with curcumin}}{OD \text{ value of curcumin}} \times 100 \quad \text{Eq. (7)}
\]
The *in vitro* curcumin loaded AFCNG release study was performed using Franz diffusion cell method. The cellulose acetate dialysis membrane (LA390, average at width 25.27 mm, average diameter 15.9 mm and capacity approximate 1.99 mL cm\(^{-1}\)) mimics human skin. The membrane was clamped between the donor and receptor compartments of the diffusion cell as reported by Mandal et al [3]. The curcumin loaded AFCNG was kept on donor compartment. The PBS was kept in the receptor compartment and it was stirred and maintained at 32±0.5ºC as similar to the normal skin temperature of human. The samples were withdrawn at different time intervals and topped with equal volume of fresh buffer. The curcumin release (%) was determined using UV vis. spectrophotometer at 420 nm.

\[
\text{Release} \% = \left( \frac{\text{OD value of sample}}{\text{OD value of AFCNG loaded with curcumin}} \right) \times 100
\]

Eq. (8)

The cytotoxicity of curcumin loaded AFCNG against B16-F10 cells was assessed. The apoptosis assay of curcumin loaded AFCNG against B16-F10 cells was examined by using DAPI staining.

**Results and Discussion**

**Amine Functionalized Cellulose Nano Gel**

**Fourier-transform infrared spectroscopy**

Fig. 3 shows the FTIR spectra of β-alanine, folic acid and FA-β-alanine-moiety. In Fig. 3 (a) the peaks at 1630 cm\(^{-1}\), 1565 cm\(^{-1}\), 1463 cm\(^{-1}\) belongs to the (–C=O) group, NH\(_2\), C-N groups of β-alanine moiety respectively. The spectrum of FA shows peaks at 1690 cm\(^{-1}\) (–C=O), and 1566 cm\(^{-1}\) (N-H primary), and 1543cm\(^{-1}\) (–C=C) indicate stretching of phenyl and pterin rings of folic acid. The peak at 1629 cm\(^{-1}\) corresponds to the amide bond of the β-
alanine conjugated with folic acid. The obtained results are in line with the earlier findings of Pradeepkumar et al [36].

Fig. 3 FTIR spectra of (a) β-Alanine, (b) Folic acid and (c) FA-β-Alanine moieties

Fig. 4 shows the FTIR spectra of MCC, CNC, OCNC, AFCNC and AFCNG. The spectra of unmodified MCC and CNC demonstrate a strong band at 3309 cm$^{-1}$, and a band at 1640 cm$^{-1}$ correspond to the stretching and bending modes of the surface hydroxyls. The peak at 2882 cm$^{-1}$ indicates the asymmetric stretching vibration of C-H in a pyranoid ring and the peak at 1034 cm$^{-1}$ belongs to the C-O of cellulose in Fig. 4 (a)-(e). Since, there is no chemical structural change from MCC to CNC, Fig. 4 (a) and (b) exhibit similar peaks. Spectrum of OCNC, Fig. 4(c) has appearance of C=O stretching of COOH at 1726 cm$^{-1}$ due to change of hydroxyl groups of CNC into COOH of OCNC. The second band at 1609 cm$^{-1}$ represents the OH bending of the adsorbed water. The disappearance of band at 1726 cm$^{-1}$ in Fig. 4 (d)
spectra reveals the successful surface amidation reaction of OCNC with FA-β-alanine moieties coupling scheme. The peak at 1598 cm\(^{-1}\) corresponds to C-N group. The peaks at 1630 cm\(^{-1}\) and 1560 cm\(^{-1}\) correspond to the Amide I and Amide II respectively. However, these peaks are not present in Fig. 4 (d) and (e). Hence, XPS study was carried out to investigate the presence of amide group. Since there is no chemical change from AFCNC to AFCNG, the spectra of Fig. 4 (d) and (e) are found to be similar.

![FTIR spectra](image)

**Fig.4 FTIR spectra of (a) MCC, (b) CNC, (c) OCNC,(d) AFCNC powders and (e) AFCNG**

**X-ray diffraction**
Fig. 5 shows X-ray diffraction patterns of MCC, CNC, OCNC, AFCNC and AFCNG. The MCC exhibits diffraction peaks at 15.24°, 16.30°, 22.84° and 34.95°. These peaks belong to β-D cellulose crystal planes (1-10), (110), (200) and (004) respectively. Analysis of XRD shows that there is no change in the patterns from Fig. 5 (a) to 5 (d). It indicates that there is no change in the crystalline nature of MCC, CNC, OCNC and AFCNC. It can be noted from that the AFCNG diffraction peaks at 15.24°, 16.30° and 34.95° were disappeared due to remove excess of solvent calcium chloride and methanol. The AFCNG was amorphous structure by using conventional stirring, centrifugation and sonication. The crystallinity of CNC, OCNC and AFCNC was 74.31%, 75.14% and 71.74%, respectively. This indicates that the modification with oxidation and amidation did not significantly change the crystalline structure and the crystallinity of CNC. In comparison, the AFCNG profile is different from AFCNC because of the amorphous nature of AFCNG as shown Fig. 5 (e). The crystallinity of AFCNG was 25.06%, which is useful for drug delivery application.

Fig. 5 XRD spectra of (a) MCC, (b) CNC, (c) OCNC, (d) AFCNC powders and (e) AFCNG
X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) was carried out to further elucidate the surface properties of sample. Fig. 6 (a) to (c) shows XPS spectra of CNC, OCNC and AFCNC respectively. XPS spectra reveal that carbon and oxygen are the predominant materials, which occur at 283.63 and 534.11eV respectively. The new peak detected in Fig. 6 (c) at 398.83eV indicates the presence of nitrogen in AFCNC. The spectra in Fig. 6 (c) and (d) are found to be similar because there is no chemical change from AFCNC to AFCNG.

Fig. 6 XPS spectra of (a) CNC, (b) OCNC, (c) AFCNC and (d) AFCNG

Zeta Potential Study

The zeta (ζ) potential was measured to understand the colloidal stability of samples as shown in Fig. 7. The ζ potential value of CNC is −23.13 mV and the OCNC is −21.53 mV. In general, The colloidal stability is considered to be stable if the zeta potential is more positive
than +30 mV or less negative than -30 mV [37]. However, in the case of nano material suspensions with extremely low ζ potential are unstable and would lead to aggregation. For cellulose nanocrystals, agglomeration will occur if the ζ potential is between +15 and -15 mV [41]. The zeta potential value of AFCNC is -59.55 mV due to the presence of carboxylic, hydroxyl and amide groups and the zeta potential value of AFCNG is found to be -53.33 mV. The study shows that AFCNC and AFCNG solutions have good colloidal stability and the particles do not agglomerate.

Fig. 7 Zeta potential value of (a) CNC, (b) OCNC, (c) AFCNC and (d) AFCNG
Particle size analysis

The particle size is one of important factors influencing the drug carrier for the targeted drug delivery [42]. The instrument based on dynamic light scattering was used to investigate the hydrodynamic size of CNC, OCNC, AFCNC and AFCNG. The histograms in Fig.8 show that maximum number of CNC particles has the diameter in the range from 47 to 95 nm with mean diameter of 74 nm. The mean diameters of OCNC particles have marginally increased from 74 nm to 82 nm and majority of particles have diameter in the range of 54 nm to 110 nm. The increase in size may be attributed to the conversion from alcohol to acid group. The histogram of AFCNC shows that the mean diameter is 80 nm with majority particles have the diameter in the range of 45 nm to 110 nm. There is no significant change in the size of nanoparticles from OCNC to AFCNC. The AFCNG has mean diameter of 74 nm with majority particles in the range of 47 nm to 96 nm. The earlier study shows that the potential carriers refers to structures of 1-100 nm size regime for drug delivery application which has effectively improved blood circulation time and enhanced extravasation rate into permeable tissues such as tumors [43]. In this experiment, the AFCNC has mean diameter and majority particle’s diameters are well within this range.
Fig. 8 Particle size distribution of (a) CNC, (b) OCNC, (c) AFCNC powder and (d) AFCNG

High-resolution scanning electron microscopy

Fig. 9 shows the High-resolution scanning electron microscope (HRSEM) micrographs of samples. The SEM image of MCC shows the deformed rod like structure due to presence of lignin and hemicellulose materials. The Fig. 9 (b) shows that spray dried CNC is nonporous and has deformed spherical shape as a result of shrinkage during spray drying. The TEMPO oxidized nanocrystals have the folded ribbon-like shape. AFCNC shows the folded ribbon-like shape as observed in the case of OCNC. AFCNG shows porous and smooth surface due to regeneration of AFCNC. The AFCNG’s network structure that mimics natural cell membrane would support the growth and transport of cells.
Fig. 9 HRSEM of (a) MCC, (b) CNC, (c) OCNC, (d) AFCNC and (e) AFCNG
Thermogravimetric analysis

Fig. 10 TGA of (a) MCC, (b) CNC, (c) OCNC, (d) AFCNC and (e) AFCNG

The thermo gravimetric curves of MCC, CNC, Tempo oxidized CNC and AFCNC are shown in Fig.10 (a) to (e) respectively. The initial weight loss occurs at around 100 °C due to the evaporation of water from the material. In the case of MCC, the second weight loss, i.e. degradation takes place at 350 °C and in the case of CNC, it takes place at 300 °C. The degradation temperature is higher for CNC due to presence of lignin and hemicelluloses. The thermal stability of OCNC is found to be lower than that of CNC. This is because of the formation of carboxylic acid groups at the C6 position of cellulose nanocrystal surfaces by the TEMPO oxidation. The second degradation of AFCNC and AFCNG happens at 240 °C. The thermo gravimetric curve of AFCNC and AFCNG indicate that the thermal stability of AFCNG is lesser than AFCNC due to its amorphous nature. It can be noted that the
degradation temperature of all the samples are more than 150 °C which is normally used for sterilization by autoclaving. The conversion of MCC to AFCNG and comparison of properties show that AFCNG has better properties compared to AFCNC and can be considered suitable for being used as carrier for drugs. Hence, other important characteristics such as water absorption and biological studies have been conducted for AFCNG.

**Water uptake**

Water uptake is an important characteristic of biomaterials and is greatly influenced by their morphology and chemical structure. The water absorption of AFCNG composite is shown in Fig.11. The AFCNG were examined at pH 4, 7 and 9. The AFCNG has higher water absorption at acidic pH compared with the neutral and basic pH. The peptide, carboxylic acid and hydroxyl groups present in the AFCNG have strong electronegative charge. At acidic pH, the positive charged water molecules get attracted easily and would be advantageous for pH responsive drug delivery application. The AFCNG with the porous, amorphous structure allows the water molecules to travel through and permeate it.

![Fig. 11 Water uptake of AFCNG](image-url)
Biodegradation

The microstructure of AFCNG was analysed for enzymatic degradation using SEM. The AFCNG was incubated in lysozyme buffer solution for five weeks. Significant difference has been noticed on AFCNG treated with lysozyme solution, which exhibits more irregular pore boundaries and begins to degrade beta 1, 4 glycosydic bond and peptide group as shown Fig. 12c that depicts the honey-comb like microstructure. The control AFCNG i.e. the sample not incubated with lysosome shows smooth and pore like structure as shown in Fig.12 (b). The degradation is found to be higher after 5 weeks. The in vitro biodegradation of AFCNG is shown in Fig. 12 (a). AFCNG was deteriorated for about 24% after one week in the presence of lysozyme and about 83% at the end of five weeks. This is due to breaking of amide bonds in AFCNG by the lysosome. The study shows that AFCNG is biodegradable and loose its weight steadily over the time. The smooth and porous surface of AFCNG becomes deformed due to the biodegradation. This AFCNG can act as carrier for biomedical applications.
Fig. 12 (a) Biodegradation at different durations, (b) AFCNG before degradation, (c) degradation after one week and (d) degradation after 5 weeks

Hemolysis Assay

Blood compatibility is crucial for successful transdermal administration and transport of drug carriers. The prepared nanogel formulation is highly biocompatible in the presence of OH, COOH and CONH groups. Hence, hemolysis study was conducted to understand the blood compatibility of AFCNG. In the present study, no hemolytic effect has been observed
visually for the AFCNG conc. of 0.4 - 2.0 mg/ml. The Fig 13 (a) shows hemolysis% of AFCNG at different concentrations and Fig 13 (b) shows the positive, negative control and AFCNG blood samples. The Fig. 13 (a) shows that hemolysis% of AFCNG samples at all concentrations is below 2%. This value is well below the limit for biomaterials according to ISO/TR 7406 [38, 44–46].

![Fig. 13 (a) Hemolysis % (b) blood samples with AFCNG](image)

The nanosize of hydrogel is able to enter into the blood vessels and negative surface charge of AFCNG is stable which are suitable for transdermal drug delivery application.

**Cytotoxicity Test and Morphological Assessment**

The cytotoxicity effect of AFCNG against the 3T3 cell lines was investigated by conducting MTT assays for 24 hours. The Fig. 14 shows the cell viability of control and AFCNG at different concentrations. No obvious cytotoxicity against 3T3 is found after 24h of treatment at all concentrations. The study indicates that AFCNG is safe and non-cytotoxic to 3T3 cells, which is an important character for the drug carriers. The cationic charges as well as the hydrophobicity of the polymer enhance the cytotoxicity [47, 48], whereas negatively surface
charged polymers reduce cytotoxicity [49–53]. In the present study, anionic surface charged and hydrophilic AFCNG is non-toxic to 3T3 cells and stable in colloidal form.

![Cell viability of AFCNG at different concentrations](image)

**Fig. 14** Cell viability of AFCNG at different concentrations

**Cellular morphology assessment by DAPI staining**

Figure 15 I and II respectively show the fluorescent microscopic image of control 3T3 cell and the cells incubated with AFCNG. In the figure (a) is bright field image (b) is image with blue filter and (c) is merging of (a) and (b). The concentration of AFCNG used in this study is 1.0 mg/ml. The figures show that there is no alteration in the cell shape even after 24h of incubation. These results indicate that the negative surface charged AFCNG is a suitable nanocarrier for drug delivery applications.
Fig. 15 Cellular morphology assessment of 3T3 by DAPI assay at 3 days (I) control 3T3 cell lines and (II) treated 3T3 cell lines (a) bright field (b) blue filter and (c) Merge

Curcumin loaded Amine Functionalized Cellulose Nano Gel

FTIR Spectroscopy and XRay Diffraction study

FTIR spectrum 16 (1b) for curcumin shows peaks at 3501 cm$^{-1}$ corresponds to phenolic OH, 1626 cm$^{-1}$ to C=O stretching, 1503 cm$^{-1}$ to aromatic C-C and 1425 cm$^{-1}$ to alkanes. FTIR spectrum 16(1c) of curcumin loaded AFC nanogel shows the peak at 3333 cm$^{-1}$ indicates presence of OH. The new peak appearing at 1503 cm$^{-1}$ in 16 (1b) and 17 (1c) corresponds to aromatic C-C groups of curcumin. This is mainly because of physical interactions that present between curcumin and AFCNG due to the hydrogen bonding interaction. The XRD pattern of curcumin 16 (2b) shows multiple peaks in the 20 range of 20-30º depicting its crystalline nature. Curcumin is a hydrophobic drug and highly crystalline in nature and inhibits its release in aqueous solution. After loading with AFCNG nanogel, the peaks of curcumin
completely disappears (Fig.16 (2c)). The dispersion of curcumin shows the successful incorporation into AFCNG. The CAFCNG is amorphous and its crystallinity is 16.61%.

Fig. 16 (1) FTIR and (2) XRD of (a) AFCNG, (b) curcumin and (c) CAFCNG

**Fluorescence Spectroscopy**

The fluorescence spectrum is used as a biomarker of molecular conjugation which provides not only the drug distribution profile inside the cells, but also the therapeutic bio imaging capabilities combined in the same molecule [32]. Fig. 17 (a) and (b) show excitation at $\lambda=453$ nm and emission at $\lambda=544$ nm for curcumin. In the Fig. 17(a) and (b), similar spectra with lower intensity is found for CAFCNG, which reveal the fluorescence quenching caused by molecular interactions between curcumin and AFCNG after the hydrogen bonding. Hence, it is confirmed that curcumin is effectively coupled with AFCNG due to the hydrogen bonding.
Fig. 17 Fluorescence Spectroscopy (a) excitation (b) emission of curcumin and curcumin loaded AFCNG

**Drug loading**

The UV spectrogram of curcumin and CAFCNG are shown in Figure 18. The drug entrapment for CAFCNG was determined using Equation 7 and it was found to be 84.37%. The result shows that curcumin is entrapped well in the AFCNG.

Fig. 18 UV spectrogram of curcumin and CAFCNG
**In vitro drug release**

The drug release study of the curcumin from CAFCNG was examined at different pH levels (4.5 and 7.4) at 32 ± 0.5 °C as shown in Fig 19 (a). The drug release percentage was calculated as per Equation 9. The drug release from the CAFCNG is 79.4% at acidic medium for 48 h due to the presence of anionic amide, carboxylic and hydroxyl groups. These groups are easy to protonate in cationic acidic pH solutions and break the carrier and produce sustained release. The protonation of amide group of CAFCNG enhances drug release. The release profile comprises simple diffusion, nanogel degradation and displacement of counter ions present in the environment. The drug release percentage of CAFCNG at pH 7.4 (physiological saline medium) is 31.4% for 48h, which indicates minimal drug release and less toxic response on normal skin. More drug release in acidic conditions is advantageous as the cancerous tissue, intracellular lysosomes and endosomes are acidic which help in active drug release from the carriers.

Fig. 19 (a) Drug release and (b) *in-vitro* cytotoxicity of CAFCNG
In vitro cytotoxicity

The cell viability of CAFCNG at different concentrations is shown in Fig.19 (b). It can be seen that CAFCNG produce toxic response to B16-F10 cell line due to the presence of the curcumin molecule and the response increases with increase in concentration. The anticancer effects of each concentration were further examined using optical microscopic imaging, as shown Fig. 8 (a) to (f). The control cells do not show change in characteristic morphology as observed in Fig. 20 (a). The cells treated with CAFCNG demonstrate notable morphological alterations in the cells such as rounding and shrinking, which are some of the defined features of apoptosis and shrinking leads to death of cells which can be found from Fig. 20 (b) to (f). The results of optical microscopic images of cell cytotoxicity confirms the MTT assay's results of cytotoxicity. Besides, the high negative surface charge of CAFCNG conjugates with the positive surface charge of cell membrane and produces high cytotoxicity to skin cancer cells via receptor mediated endocytosis.
Fig. 20 Optical microscopic image of CAFCNG on B16-F10 cell lines, (a) Control, (b) CAFCNG at conc. 0.2 mg/ml, (c) 0.4 mg/ml, (d) 0.6 mg/ml, (e) 0.8 mg/ml and (f) 1.0 mg/ml
Apoptosis morphology assessment by DAPI staining

The apoptotic potential of CAFCNG on B16-F10 cells was assessed by employing DAPI staining as shown in Fig. 21. The results revealed that the untreated cells (control) exhibit uniform fluorescence in their cytoplasm as well as nuclei, without any signs of fragmentation or condensation. However, after 24 hours of treatment with CAFCNG, the cells display noticeable shrinkage and visibly apoptotic nuclei, with blebbing and apoptotic bodies. The bright field images of the cells undergoing typical alterations, marked with arrow signs, demonstrate the morphological changes occurring during the cell death process. While free curcumin is impeded from accumulating inside the cells due to ATP-dependent efflux pumps on the cell membrane, CAFCNG can be taken up by folate receptor-mediated endocytosis. Moreover, when curcumin is loaded into nanogels, it can escape from the efflux pumps, accumulate in the cells, and rapidly induce apoptosis [7]. The observed results highlight the potential of CAFCNG as an efficient targeted drug delivery system for transdermal applications.
Conclusions

In this study, the amine-functionalized cellulose nanogel (AFCNG) was synthesized and formulated using controlled regeneration approach. The AFCNG was found to have hydrophilic and blood-compatible nature, with very low cytotoxicity to 3T3 cells and good biodegradability. Moreover, the AFCNG demonstrated high loading capacity for the anticancer drug curcumin, and its surface groups enabled it to exhibit high release at acidic medium than neutral, which can selectively accumulate on malignant tumor tissues and inhibit tumor growth. The cellular uptake experiments confirmed that the AFCNG can deliver anticancer drugs into the cells by receptor-mediated endocytosis, and its negative surface charge can bind to tumor cells with high selectivity. Overall, the findings suggest that
CAFCNG is a promising candidate for skin cancer treatment as an effective anticancer drug carrier.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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