Modified Fluorescent PEG-Based Carrier System for \textit{In-vitro} Imaging

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Abstract. A facile, one-pot synthesis of polymer based fluorescent coating agent was developed. Polyethylene glycol (PEG), known to be biocompatible and biodegradable, has been modified with ascorbic acid (AA) to yield modified fluorescent PEG based carrier system (PEGAA). The fluorescence obtained was tuned using varying concentrations of ascorbic acid. With increasing concentration of ascorbic acid, a red shift in fluorescence was observed which could be utilized for effective \textit{in vitro} imaging. The cellular uptake studies reveal the potential use of this novel material for bioimaging applications.

Keywords: Polyethylene glycol, PEGAA, Bioimaging

1. Introduction

Owing to the \textit{in vivo} compatibility and degradability, biodegradable polymers are widely opted for various biomedical applications such as tissue engineering, drug delivery, gene delivery etc. They have been used as coating material for implants to eliminate the direct interaction between implants and biomolecules in order to reduce toxicity. [1-2] Biocompatible carrier systems, especially polymeric carrier systems are often used in drug delivery to reduce drug associated toxicity. The type and nature of the polymeric coating up on the drug molecule, influences the particle size, drug release and the physiological circulation time. [3-4] Among polymeric carrier systems, low molecular weight Polyethylene glycol (PEG) molecules exhibiting enhanced biocompatibility and optical properties are well preferred for diagnostic applications. [5] PEG, well recognized for its high biocompatibility and resistance to opsonization due to inherent immune response, has paved the way for its use in the medical research field as efficient coating agents. [6] Additionally, PEG coating provides an enhanced circulation time under physiological conditions when compared to other polymeric coatings. The enhanced circulation time increases the enrichment of drug entities within the tumour site through enhanced permeability and retention (EPR) effect. Literature reports indicated that PEG of varying molecular weights, or their conjugates such as lipo-polymer carrier systems, are well considered for encapsulation of drugs. [7] PEG-based carrier systems in conjugation with fluorescent dyes are used for cancer/tumour diagnosis to eliminate the toxic side-effects of these dye molecules. Though the complex conjugation results in reduced dye toxicity, it fails to control photo-bleaching and particle size. Moreover, the complex coating over dye along with photo-bleaching deduces the fluorescence...
signal strength leading it to poor choice for bioimaging. [8] Further, fluorescing metallic nanoclusters such as gold nanoclusters are investigated for their better bio-imaging properties compared to chemical dyes. The surface interaction of these particles with biomolecules changes the hydrophobicity-hydrophilicity balance leading to aggregation followed by fluorescence quenching. [9,10] In our previous work, we have reported the synthesis of fluorescently modified PEG to overcome the shortcomings of current fluorescent carrier systems. [11] The biocompatible PEG exhibited excellent fluorescence upon heating at optimum conditions. This modified fluorescent PEG, termed as FL PEG, possessed good biocompatibility, enhanced circulation time and tunable fluorescence. Though in vitro bioimaging showed a tunable fluorescence, the fluorescent emission intensity of FL PEG was found to be maximum when it was excited with a wavelength of 450 nm (blue light). The corresponding emission wavelength was obtained at 515 nm (Green emission).

This work is aimed to improve the fluorescence intensity throughout the visible spectra. PEG of molecular weight 300 (PEG M.w 300), used in the synthesis of PEGAA, owing to its better biocompatibility. In order to tune the emission intensity, Ascorbic acid was used to modify PEG resulting in formation of PEGAA. The absorbance spectra and fluorescence emission spectra of the particle recorded, showed the tunability of inherent fluorescence of the novel carrier system. Further, in vitro bioimaging studies were performed to authenticate the bioimaging application of the developed complex.

2. Materials and Methods

2.1. Materials
Polyethylene glycol (PEG, Mw:300) and Ascorbic acid were purchased from SRL (India). Phosphate buffer pH 7.0, Trypsin-EDTA, DMEM, Bovine Serum (US origin. All chemicals were reagent grade and used as received. Ultra-pure de-ionized water (Milli Q) was used for all solution preparations and experiments.

2.2. Synthesis of PEGAA sample:
PEG (Mw :300) was heated at 95 °C for about 30 minutes, followed by the addition of predetermined concentrations of ascorbic acid (5 mg, 10 mg, 20 mg and 30 mg). Upon further heating for 5-7 minutes, the bright yellow coloured solution obtained was monitored under a UV lamp of 365 nm. The green fluorescence observed indicated the formation of PEGAA complex.

2.3. Characterization:
The PEGA sample was evaluated for its fluorescence under UV lamp (365 nm, Analytik Jena, CA, U.S.A) as initial characterization. Excitation and emission properties of PEGAA samples were analyzed using fluorescence spectroscopy (RF 6000, Shimadzu, Japan). The UV-Visible absorbance was analyzed by UV-visible spectroscopy (UV 1800, Shimadzu, Japan).

2.4. In-vitro studies

2.4.1 Cell culture & maintenance: Human breast cancer cell lines (MCF-7) were obtained from CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India. The cell line was cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-Glutamine, and 100 U/ml penicillin/streptomycin and were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ under sterile conditions.

2.4.3 In-vitro imaging: Human breast cancer cells (MCF-7) were used for bio imaging of PEGAA samples. Briefly, cells were seeded in a 24 well plate, 24 hours prior to the treatment, at a density of 5×10⁴ cells per well. On the following day, the cells were treated with PEGAA prepared with varying
ascorbic acid concentration (5 mg, 10 mg, 20 mg and 30 mg). The cells were incubated with PEGAA samples for 24 hours. After the incubation period, the culture media was removed, cells were washed twice with PBS and the fluorescence was recorded with fluorescent microscopy (Olympus CKX-53, U.S.A). [12,13]

3. Results and Discussion

3.1. Synthesis and characterization of PEGAA samples

Upon heating PEG in presence of ascorbic acid, the solution turned bright yellow in color and was initially characterized for its fluorescence. The samples showed a green emission when showered with UV-lamp of wavelength 365 nm. Figure 1a shows the bright light imaging of PEGAA prepared with various concentrations of AA (5 mg/ml, 10 mg/ml, 20 mg/ml and 30 mg/ml). It was observed that with increase in the concentration of ascorbic acid from 5 mg/ml to 30 mg/ml, colour of the solutions turned darker. Figure 1b shows the corresponding fluorescence of PEGAA samples. The fluorescence observed under the UV lamp was validated with fluorescence spectroscopy. The normalized fluorescence spectra (Figure 1c) shows the shift in emission peak as the concentration of AA increases.

Figure 1: Characterization of PEGAA sample: Digital photographic image under a) Bright light, b) UV lamp of 365 nm and c) Normalized emission spectra for the excitation 365 nm.

UV Visible absorption spectroscopy was further used to evaluate the absorbance (Figure 2a). The absorbance spectra obtained were similar to that of our previous work. [11] Black solid line representing the UV absorbance of PEG diluted against water was considered as experimental control. The absorbance spectra revealed formation of a broad spectra, in place of the characteristic absorbance peak, at high frequency region (190-400 nm). The UV Vis absorbance spectra indicates a red shift in absorbance with increase in the concentration of AA. This could be attributed to the formation of molecular aggregates caused by elevated temperature. Due to the absence of characteristic UV Vis absorbance peak, fluorescence of the sample was analyzed with excitation and emission scan. Both Emission (Figure 2b) and Excitation (Figure 2c) spectra showed a red shift in peaks as the concentration of AA increases.
An experiment was performed to analyze the fluorescence behaviour of the PEGAA sample. PEGAA containing 10 mg/ml of AA, having a better emission intensity, was taken for this experiment. 1 ml of the sample was added with equal volume of nHexane. nHexane was used accounting for its immiscibility and lower density when compared with the PEGAA sample. The emission spectra showed (red solid line) a slight increase in intensity when compared to the control sample (green solid line). Further, addition of nHexane (2 ml) showed a significant decrease in fluorescence (Figure 3a). Similar effect was also observed in the corresponding excitation spectra (Figure 3b). The experimental results indicate that though the PEGAA samples can obtain an increased intensity through aggregation induced emission (AIE), further molecular aggregation can result in quenching of the fluorescence (AIQ).

3.2. In vitro studies

A qualitative in vitro bioimaging of PEGAA samples was evaluated in Human breast cancer cells (MCF-7) cells. Upon 24 hours of incubation, the cells treated with predetermined concentration of PEGAA were analyzed for cellular uptake using fluorescence microscopy.

Figure 4 shows the fluorescence of PEGAA samples of various concentrations of ascorbic acid. The biocompatible, self-fluorescing complex exhibited fluorescence under green light as shown. The cells were able to exhibit fluorescence at an ascorbic acid concentration of 10 mg/ml. A faint red fluorescence could be observed when the sample was excited using green light. A better fluorescence due to cellular uptake was visible for the ascorbic acid concentration of 30 mg/ml. In all the cases of varying ascorbic acid concentrations, the cell morphology remains unaffected, indicating the biocompatible nature of PEGAA samples. This further points out that the material has the potential to replace the existing non fluorescent carrier systems and potentially toxic chemical dyes.

Figure 2: Spectral analysis: a) UV Visible Absorbance spectra b) Normalized Emission spectra and c) Normalized excitation spectra of PEGAA samples upon various concentrations of Ascorbic acid (5 mg/ml, 10 mg/ml, 20 mg/ml and 30 mg/ml).

Figure 3: Fluorescence analysis: a) Emission and b) Excitation spectra.
Figure 4: *In vitro* bioimaging (10x magnification) of PEGAA for various AA concentrations.

4. Conclusion

In summary, we have developed a facile one-pot approach for the synthesis of PEGAA using PEG and ascorbic acid. The spectral analysis confirms the shift in self fluorescence of PEGAA from green to yellow upon varying concentrations of ascorbic acid. *In vitro* bioimaging studies reveal the possibility of its application as fluorescent encapsulating agent for medical devices and toxic drug molecules.

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