Colonic Carcinoma Diagnosis using Chitosan Nanoparticles Based on the Optical Properties.

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Abstract: Medical diagnosis using optical techniques and contrast agents is a promising method where it is safe and unexpensive technique. Every tissue can be distinguished by its optical absorption and scattering properties that are related to many physiological changes and it is a sign for cancerous cells. Characterizing the light propagation in the human tissues is a vital issue in early cancer diagnosis for more effective therapeutic. In this work, the glowing effect of chitosan nanoparticles has been observed. Also, the light propagation in each of colon cancer (Caco-2 cell line) and normal cells (WI-38 cell line) at 650 nm and 808 nm in the absence and in the presence of chitosan nanoparticles was studied to study its effect in differentiate the cancer cells from the normal cells. Chitosan nanoparticles were characterized by the dynamic light scattering and transmission electronic Microscope (TEM). A Monte-Carlo simulation model was applied to obtain spatially resolved steady state diffuse reflectance measurements for each of the examined cells. Furthermore, the optical fluence rate distribution at the tissue surface were used to reconstruct the image using the diffuse equation using the finite element method. Chitosan nanoparticles appeared its glowing effect. The proposed diffuse reflectance curves and fluence rate images show different features regarding for each of Caco-2 cell line and WI-38 cell line that promises to be effective in medical diagnosis.

Key words: Monte-Carlo simulation of Light Propagation, Tissue Optical Parameters, Diffusion Equation, Finite Element Method

1. Introduction
Colon cancer is a very common type of cancer that has almost no symptoms in its initial stage; therefore, early detection can greatly help in treatment of such type of cancer [1]. The available cancer diagnosing techniques such as colonoscopy has some limitations including low sensitivity and high cost [2]. Therefore, there is a need to develop more efficient prognosis and diagnosis methods. Recently, the use of biomarkers and contrast agents showed a remarkable success in colon cancer screening methodologies [3]. Besides their role in cell imaging and drug delivery, Chitosan
nanoparticles (C NPs) have also photonic applications and can help in cancer management. It also played an important role in the early detection of colorectal cancer [4] [5].

Any biological tissue can be characterized by its optical absorption and scattering properties. The optical parameters are considered a very efficient sign for tissue health and function, therefore normal tissue cells have different values of absorption and scattering coefficients rather than cancer cells [6]. Tissue absorption and scattering coefficients are basically calculated from experimental measurements of the diffused light. Tissue diffuse reflectance and transmission can be measured using integrating sphere based techniques [7] or distance-detector configurations [8] [9].

In this study, colon cancer cells (Caco 2 cells) were differentiated from normal cell (WI 38 cells) depending on their optical properties using 650 nm and 808 nm laser irradiation. The optical parameters were determined using distant-detector measurements and Kubelka-Munk transport model. The distribution of the fluence rate at the sample surface was also investigated at the same wavelengths using the finite element solution of the diffusion equation showing different distribution for each sample. An optical contrast agent “C NPs” were added to the samples and the glowing effect has been observed under inverted microscope during exposing the NIR laser.

2. Material and Methods
Managing the cancer cells from the normal cells using NIR laser was based on the distinction between the tissue optical parameters then the optical fluence rate imaging. The following steps brief the assay, first, C NPs synthesized and characterized by the dynamic light scattering and transmission electronic microscope (TEM). Second, the diffused light measurement (Reflectance and Transmittance) was measured for each of normal cells (WI 38 cell line) and cancer cells (Caco2 cell line) at 650 nm and 808 nm with and without injected the C NPs. Third, the optical parameters were calculated using Kubelka-Munk model and the fluence rate images were determined using a finite element solution of the diffusion equation.

2.1. Chitosan nanoparticles (C NPs) preparation
Chitosan (medium-molecular-weight (161.1MW)) and the Tripolyphosphate (TPP) ((Na5P3O10, with a molecular weight: 367.86)) were obtained from Oxford company, India. One hundred milligram of Chitosan was dissolved in one hundred milliliters of 1% diluted acetic acid under continuous magnetic stirring at 2000 rpm for two hours to get the cationic solution. Thirty-three milligram of TPP dissolved into ten milliliters of distilled water. The TPP solution dropped into the cationic solution under magnetic stirring at 2000 rpm for 45 min in the room temperature, the clear solution gradually became milky (C NPs formed). Then, C NPs solution was dialyzed with 12000 rpm centrifuge for twenty minutes. The sponges were prepared by the direct freeze-dry of C NPs dispersion.

2.2. Physicochemical characterization of the C NPs
The surface charge and the hydrodynamic diameter of the C NPs were determined using the Dynamic Light Scattering (DLS), (ZS-ZEN (Malvern Instruments) Co., UK), (Specification: Diameter range (0.6:6000 nm), Zeta potential range (-200 to 200 mV)). High-Resolution Electronic Transmission Microscope (HR-TEM, Tecnai G20, FEI, Eindhoven, Netherlands) was used to determine the morphology of the NPs. The imaging applied in two stages, first stage, the C NPs solution were pipetted up and down to be suspended. Second stage, 2-5 μL drops of solutions were mounted on carbon-coated 400-mesh copper grids, the specimens were left to dry for 2 min.
2.3. cell line
Caco 2 cell line (colon cancer) and WI 38 cell line (Normal cells) were purchased from the National Cancer Institute, Cairo university, Egypt.

2.4. Calculating the optical parameters
The optical scattering and absorption parameters of normal cells (WI38 cell) and colon cancerous (Caco2 cells) were determined at 650 nm and 808 nm laser irradiation. The experiment was performed with and without C NPs injection. Diffuse reflection and transmission of the studied sample was measured using the experimental setup presented in Figure 1. The laser radiation probes the sample and diffused light is measured using photodetector connected to a spectrometer through an optical fiber. The collected data are then sent to computer for further analysis.

Figure 1. Schematic diagram for diffused light measurement optical setup

Kubelka-Munk model is utilized for estimating the absorption coefficient $\mu_a$ and reduced scattering coefficient $\mu'_a$ of the studied samples. It assumes two Kubelka-Munk coefficients that are related to diffuse reflectance and transmittance as follows:

$$a = \frac{1 + R_d^2 - T_d^2}{2R_d}$$  
$$b = \sqrt{a^2 - 1}$$  

The two coefficients $a$ and $b$ are related to the fraction of loss in flux due to absorption $A$ fraction due to scattering $S$, where $A$ and $S$ are related to $R_d$ and $T_d$ by:

$$S = \frac{1}{bd} \ln \left[ \frac{1 - R_d(a - b)}{T_d} \right],$$  
$$A = (a - 1)S$$  

Then the scattering and absorption coefficients are expressed as:

$$A = 2\mu_a$$
\[
S = \frac{3}{4} \mu_s(1 - g) - \frac{1}{4} \mu_a
\]  
(6)

where \( \mu_s(1 - g) = \mu_s \) is called the reduced scattering coefficient.

2.5. Optical fluence rate determination
The diffusion equation considers an approximation to the radiative transport equation “RTE” which used to describe light propagation in biological tissues. The fluence rate distribution at the sample’s surface was determined using a finite element solution of the following diffusion equation.

\[
\frac{\partial \Phi(\vec{r}, t)}{\partial t} + \mu_a \Phi(\vec{r}, t) - \nabla \cdot [D \nabla \Phi(\vec{r}, t)] = S(\vec{r}, t)
\]  
(7)

where \( D \) is the diffusion coefficient which is defined as \( D = \frac{1}{3(\mu_s + \mu_a)} \), \( \Phi(\vec{r}, t) \) is the fluence rate (in W/cm²) and \( S(\vec{r}, t) \) is the source term (in W/cm³ sr).

2.6. Observing the Glowing Light of the synthesized nanoparticle during the photomicrographs
The glowing effect of the C NPs were observed under the inverted microscope (Zeiss Axio Vert.A1; Zeiss, Gottingen, Germany) at 40 magnifications by photographing Cacao 2 cells which injected with C NPs and exposed to the NIR laser (650 nm) during the cells imaging using a digital camera.

3. Results and discussion
3.1. Physicochemical characterization of the C NPs
The peak diameter size which measured using the DLS was 331 nm. the zeta potential (ZP) was 49.8mV. The results showed the stability of the synthesized NPs because the zeta potential charge is more than 30 mV which prevents the particles aggregation as reported by Gan et al. [10]. TEM measurements of the C NPs were represented figure 2. The mean diameter size was 75 nm (-50 nm). This difference in the measurements between DLS and the TEM measurements due to the light scattering, which leads to shifting the measured particles size towards larger values as reported by Souza et al. [11].

![Figure 2. TEM measurements](image)

3.2. Optical parameters estimation
The measurements of reflectance and transmittance were determined using (a STDFSM digital fiber spectrometer). All experiments have been read with spatial step and continue to scan up to 20°. Diffuse reflectance (Rd), Diffuse transmittance (Td), the collimated transmittance (Tc) measurements are presented in table 1 and table 2. The results show difference in the reflectance and transmittance values between colon carcinoma (Caco 2 cells) and normal tissue (WI38 cells). The measured values of total diffuse reflectance and transmittance were introduced to Kubelka–
Munk mathematical model and Bouguer-Beer-Lambert law to estimate the optical parameters (absorption coefficient ($\mu_a$), scattering coefficient ($\mu_s$), and anisotropy ($g$)). The obtained results were presented in table 3 and table 4. The results show different in the measured parameters between the Caco2 cells and the WI38 cells. The values which extracted by 650 nm were more effected in case of NPs injection where there were great differences in the measurement conversely the cells irradiated with 808 nm. The optical properties of a tissue grow up both diagnostic and therapeutic applications for cancer management using the laser. The ability of light to penetrate a tissue, interrogate the tissue components, then escape the tissue for detection is a key to diagnostic applications. The ability of light to penetrate a tissue and deposit energy via the optical absorption properties of the tissue is a key to therapeutic applications. Hence, specifying the optical properties of a tissue is a key step toward detect the cancer location and inject it with the therapeutic drug.

Table 1: Reflectance and transmittance measurements without injected chitosan NPs.

| Measurements | Wave length | Caco 2 cells | WI 38 cells |
|--------------|-------------|--------------|-------------|
| Rd           | 650         | 0.1063       | 0.1128      |
|              | 808         | 0.2245       | 0.1250      |
| Td           | 650         | 0.3194       | 0.5977      |
|              | 808         | 0.6935       | 0.6024      |
| Tc           | 650         | 0.1515       | 0.2515      |
|              | 808         | 0.3726       | 0.2115      |

Table 2: Reflectance and transmittance measurements with injected chitosan NPs.

| Measurements | Wave length | Caco 2 cells | WI 38 cells |
|--------------|-------------|--------------|-------------|
| Rd           | 650         | 0.0516       | 0.0129      |
|              | 808         | 0.3558       | 0.0453      |
| Td           | 650         | 0.7505       | 0.2311      |
|              | 808         | 0.2697       | 0.2763      |
| Tc           | 650         | 0.3748       | 0.1006      |
|              | 808         | 0.1334       | 0.1169      |

Table 3: Optical parameters at 650 nm.

| Sample       | Nanoparticles | $\mu_a (cm^{-1})$ | $\mu_s (cm^{-1})$ | g     |
|--------------|---------------|------------------|------------------|-------|
| Caco 2 cells | -             | 4.447            | 14.425           | 0.6464|
|              | C NPs         | 1.105            | 8.708            | 0.8538|
| WI 38 cells  | -             | 1.727            | 12.07            | 0.755 |
|              | C NPs         | 7.127            | 15.839           | 0.8164|

3.3. Optical fluence rate distribution

The optical fluence rate distribution were reconstructed for each of the colon cancerous (Caco-2 cells) and normal cells (WI-38 cell) with and without NPs injection. the fluence rate images are represented in figure 3, 4, 5 and 6.
Table 4: Optical parameters at 808 nm.

| Sample      | Nanoparticles | $\mu_s$ (cm$^{-1}$) | $\mu_c$ (cm$^{-1}$) | g   |
|-------------|---------------|----------------------|----------------------|-----|
| Caco 2 cells | -             | 0.420                | 9.453                | 0.5414 |
| C NPs       | 2.575         | 17.569               |                      | 0.3694 |
| WI 38 cells | -             | 1.603                | 13.927               | 0.7703 |
| C NPs       | 0.5820        | 15.645               |                      | 0.7684 |

Figure 3: Optical fluence rate distribution at 650 nm for (A) WI38 cells, (B) Caco2 cells.

Figure 4: Optical fluence rate distribution at 650 nm for C-TPP NPs for (A) WI38 cells, (B) Caco2 cells.

Figure 5: Optical fluence rate distribution at 808 nm for (A) WI38 cells, (B) Caco2 cells.

Figure 6: Optical fluence rate distribution at 650 nm for C-TPP NPs for (A) WI38 cells, (B) Caco2 cells.

The results appeared different fluency rate distributions on the caco2 cells compared to WI 38 cells in case of irradiation the cells with both 650 nm and 808nm. Also, the optical fluence rate distributions were different in case of NPs injection. At 650 nm, the maximum value of log($\Phi$) is 1.11 for WI38 cells and 1.69 at Caco-2 cells while this value is changed to 1.95 at 635 nm and 1.18 at 808 nm. The minimum value of log($\Phi$) is also changed regarding the sample condition giving values of -2.3 for WI38 cells and -0.28 for Caco-2 cells at 635 nm while at 808 nm, while the minimum value of log($\Phi$) was -1.66 and -9.3 in (WI-38 cell) and (Caco-2 cells) respectively. The difference in the fluence rate distribution is considered diagnostic indicator.
3.4. Observing the glowing light of the synthesized nanoparticle during the photomicrographs

Figure 4 showed the optical tomography for the Caco 2 cells which photographed under the inverted microscope. Caco 2 cells which injected with C NPs and exposed to NIR during the cell imaging appeared glowing effect. These results agree with Yi, X. [12] who evaluate the Near-infrared fluorescent effect in cancer detection and Marpu, S.B.[13] who employee the C NPs in clinical imaging and the photonic applications. Also, Agrawal, P.[14] operates the C NPs and NIR laser in the molecular imaging where it can penetrate the tissue for several centimeters [15] as Erhan, I. reported.

Figure 7. Representative the glowing effect of the C NPs on Caco 2 cells which photographed under the inverted microscope during exposing laser with 650 nm. Scale bar 20 μm.

Conclusion

In conclusion, a stable C NPs were prepared successfully in nano-form using ionic gelation methods. Reflectance and transmittance measurements of the cells were obtained using a STDFS digital fiber spectrometer which irradiated at 650 nm and 808 nm. The light propagation in normal and colon cell lines have been investigated using Monte-Carlo simulation and diffusion equation. The results showed different in reflectance and transmittance measurements between the normal and the Colon cells. Images representing the optical fluence distribution at the surface of the cell lines have been obtained using the finite element solution of the diffusion equation. The resultant images provided some observable discriminations between normal and Colon cells.

The cells which injected with C NPs and exposed to NIR during the cell imaging appeared glowing effect. Those results are significant for differentiate the cancer cells from the normal cells and will be helpful in early cancer diagnosis and therapeutic procedures through many medical applications such as photodynamic therapy and biostimulation.

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