

Chapter 5

Spectroscopic Techniques Used in Nanomedicine

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In this chapter we propose to report several optical spectroscopy techniques and their Nanomedicine applications (1). The overview of spectroscopy requires clear distinction between absorption and emission. When electromagnetic radiation is in contact with a sample, the photons are absorbed and the molecules of the atoms suffer a transition from the ground state to the excited state. The plot obtained from the absorbance values according to the wavelengths represents the absorbance spectrum. The transition from the excited state to the ground state implies energy emission, the basic principle in emission spectroscopy (2).

UV-Vis Spectroscopy

Due to the property of molecules or ions to absorb electromagnetic radiation in the UV and visible domain, 4 types of transition in the configuration of valence electrons may be evidenced. The most important transitions are $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ through the implication of functional groups. Chromophores are functional groups and bands which are able to induce the increase in absorption of a substance. Furthermore, transitions occurred in metal ions may lead to a visible color change, which is the principle of the qualitative detection. UV-vis spectroscopy may be also performed in quantitative manner, based on

Bouguer-Lambert-Beer law and on the parameters of attenuation of incident radiation- transmittance and absorbance, directly dependent on the concentration of the analyte (2, 3).

In order to obtain novel eco-friendly nanomaterials, Ahmad A et al used *Rhodococcus* sp., Actinomycetales order, which was cultured in specific medium. After the separation of the mycelial mass and a washing step, the biomaterial was resuspended in a HAuCl_4 solution for 24 hours. UV/VIS spectra were performed before and after resuspension. Whilst the spectrum before immersion showed no evident absorption, the one obtained after contains a detached peak at around 540 nm. Considering that gold nanoparticles (GNPs) have a maximum absorption between 520 and 580 nm according to the literature (4), the result asserts the presence of GNPs aggregates. On a macroscopic scale, it was observed that the colour of the mixture changed in intense purple after AuCl_4^- ions were added, which indicates that Au ions were reduced. Moreover, the HAuCl_4 solution is transparent. Taking together, it was emphasized that the formation of GNP take place within the cells. The observation is strengthened by the UV/VIS spectrum of the HAuCl_4 solution after the bioreaction because the absorption at approximately 540 nm was absent. The intracellular synthesis of GNP was demonstrated and the result was completed by another techniques to validate the precise localization of GNPs, which was founded to be on the cell wall and in the cytoplasmic membrane (5).

Previously, a similar approach was performed by Mukherjee P et al by culturing a fungus, *Verticillium*. The biomass was obtained and immersed in aqueous AgNO_3 solution for 72 hours. UV-vis spectroscopy was performed before and after the reaction and it was suggested the presence of a silver nanoparticle (SNP) aggregate, taking into consideration the presence of the absorption peak around 450 nm and the known maximum absorption of SNP to

be between 380 and 450 nm. The intracellular reduction of Ag^+ ions was suggested by the color conversion into brown and by the UV-vis spectrum performed before the completion of the reaction. It is of particular importance that the formation of silver sulfide nanoparticles was excluded by the characteristic aspect of the spectra. The novel SNPs and GNPs may be further used for different purposes, according to their electronical, optical, chemical and physical properties (6).

In order to develop an accessible and non-expensive diagnosis method for cancer, El- Sayed HI and co-workers designed GNPs coated with anti-EGFR antibody and the payload was added into HaCaT cells (nonmalignant cell line), respectively into HOC and HSC (malignant cell line) and compared to the uptake of colloidal gold in the same cell lines. Scattering images were performed based on the surface plasmon resonance (SPR) property of GNPs and absorption spectra were recorded. It was revealed that single GNPs form aggregates into cells, finding sustained by the bands observed around 700 nm. In comparison, no bands were observed in the absorption spectra of the conjugated nanoparticles. Moreover, the adroitness to perform specific binding was observed on malignant cancerous cells, due to the overexpression of EGFR molecules in cancerous cells. The binding process was increased by 6 and 7 folds when compared to nonmalignant cells. The results were straightened by the scattering imaging results. Because of the facility of the tools proposed, SPR spectroscopy and scattering imaging may be used in cancer diagnosis as fast and low-cost method (7).

Drug delivery systems are consisting of tremendous designs forms. GNPs polymers were conjugated with folate-PEG-thiol to deliver with high specificity doxorubicin by Banu H et al. UV-vis spectrum has been recorded before and after functionalization and the results were according to the visible change of

colour in reddish brown. Before coating with folate-PEG-thiol a maximum peak was registered at around 540 nm. After the conjugation process, the increase in dimensions of the particles were suggested by rising the peak at 560 nm (8).

Several colorimetric methods have been developed, starting from the advantages offered by UV-vis spectroscopy: ELISA (enzyme linked immunosorbent assay) (9), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (10).

Mosmann T provided a quantitative assay to evaluate cell survival. The method is based on the capacity of living mitochondria to form formazan compound from MTT. The formed purple compound has an absorption peak at 570 nm. (10) Several tetrazolium assays were developed, starting from the limitations observed in metabolizing the MTT by different cell types. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was found to be well metabolized, especially by mouse 3T3 fibroblasts and human colon tumor cells, in the presence of PMS-phenazine methosulfate (11). XTT- 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide was also tested as an alternative to MTT with a well enhancement of cellular reduction of tetrazolium salts (12).

Tuzlakoglu K and co-workers developed a combined scaffold by mixing micro and nanofibers with a payload of human osteoblast-like osteosarcoma and rat bone marrow stromal cells. MTS assay was performed to evaluate cell viability after one and two weeks and it was observed a marked cell proliferation by increasing the growth rate. The authors concluded that the nano/micro scaffold promises to be an achievable material for bone tissue engineering (13). Nanoparticles designed by Anitha A et al based on chitosan (CS)- O-carboxymethyl CS and N,O-carboxymethyl CS- were added on MCF-7 cells and MTT assay revealed no modification in cell growth rate, upholding the

absence of cytotoxicity of the chitosan based nanoparticles (14). Nano-hydroxyapatite/chitosan scaffolds were seeded with preosteoblastic cells and MTT revealed significant increase in cell proliferation suggesting also a suitable strategy for bone loss management (Kong L et al (15)).

Despite the fact that tetrazolium salts assays are currently wide-used, several observations have been reported. It was revealed that superoxide anions (SOD) interfere with tetrazolium reduction and MTT or XTT assays may lead to a misinterpretation of cell toxicity. Moreover, nano-TiO₂ induce SOD formation and applying MTT/XTT assays to evaluate cell survival is, therefore, wildly inaccurate (16). In astrocytes and HeLa cells subjected to mesoporous silica nanoparticles (MSN) endocytosis, the stimulation of exocytosis of formazan was observed and it was correlated with the overestimation of MSN cytotoxicity (17). Thus, researchers should use another cell viability techniques, as ATP assay concept, currently wide used (18).

Enzyme linked immunosorbent assay has been reported by Perlmann P and Engvall E, as a quantitative method for determination of rabbit IgG (9). The method is based on the principle of antigen-antibody interactions and it is currently wide-used especially in diagnosis. The antigen or antibody is fixed on a solid plate and then is added the liquid- phase containing the particle needed to be quantified, which will specific bind to the antibody or antigen fixed. Then, another antibody is added to bind to the analyzed molecules with an enzyme, usually horseradish peroxidase (HRP). After adding the substrate for enzyme, it occurs a color reaction, readable by spectrophotometer.

The applications of ELISA in nanotechnology field is tremendous. Vast nanoparticles have been validated. IL-10, IL-12 and TNF- α were determined by ELISA after mice infected with *Paracoccidioides brasiliensis* were treated with nanoparticles loaded with Amphotericin B (19).

The induction of bone formation by silica nanoparticles with a fluorescent core-shell was emphasized by quantitation of carboxy-terminal telopeptide of type I collagen and osteocalcin, whilst creatinine, alanine aminotransferase and TNF α were determined as markers for organ function. It was revealed that the proposed nanoparticles enhance bone formation without inducing organ damages. (20)

In order to improve the results obtained by ELISA, Ambrosi A et al used GNP as potential carrier-agents for anti-CA15-3-HRP, which binds to CA15-3 plasmatic breast cancer marker. The nanoparticles were characterized by UV-vis and TEM. To prevent the formation of aggregates, UV-vis was performed to determine the optimal concentration of anti-CA15-3-HRP which may be bound to GNP. When the optimization was completed, the novel assay was compared to classic ELISA assay and it was observed that the presence of GNP significantly increase the sensitivity (21).

Raman spectroscopy implies light scattering with loss or gain of energy-inelastic scattering (Raman scattering) (22). It is a vibrational spectroscopy method and the spectrum obtained from the difference between the wavelength of the scattered and incident light is consisting of characteristic bands (23), which are usually complementary to IR spectroscopy bands. The most used technique is Stokes Raman, in which the transition from ground to excited state is followed by energy emission to a superior vibrational energy level than the initial point. The phenomenon is more likely to happen because at normal conditions molecules are usually in the ground state. In comparison, anti-Stokes Raman spectroscopy relies on the transition from a higher energy level to the ground state. (24)

Qian X et al employed a novel technique for spectroscopic detection using GNPs functionalized with ScFv B10, a specific antibody fragment for EGFR.

GNPs with and without PEG layer were analyzed by Raman spectra under different medium conditions: concentrated salts, strong acids and bases and different organic solvents and it was established that PEG-coated nanoparticles exhibit a higher stability. The nanocomposites were incubated with an EGFR positive and an EGFR negative cell lines (Tu686 and NCI- H550 respectively) and surface-enhanced Raman scattering (SERS) spectra have been recorded to emphasize the binding specificity. Strong SERS bands were observed when ScFv-conjugated particles were incubated with EGFR positive cells. In contrast, strong signals could not be emphasized when EGFR negative cells were incubated with the proposed nanosystem, suggesting that the binding process is based on specific affinity. In vivo testing was performed for mice inoculated with Tu686 cells and injected with conjugated or non-conjugated GNPs. Strong signals have been emphasized with a gradually accumulation of nanoparticles in the tumor, suggesting that ScFv-GNPs may be used as key agents for in vivo targeting and SERS analyses (25).

In order to improve tip-enhanced Raman spectroscopy (TERS), the Au tip is replaced by a GNPs layer. The nanoparticles employed were coated with silica or with alumina. Li JF et al established the aspects of their novel technique-shell-isolated nanoparticle-enhanced Raman spectroscopy (SHINERS)- which has a higher sensitivity. The method has been applied to emphasize the Pt-H signal, on Pt(111) when a strong band may be observed when silica/GNPs are used. The silica shell was replaced with an Al₂O₃ coat and no notable changes in the recorded spectra have been observed. SHINERS may be used successfully in semi-conductor industry, due to the capability to emphasize the Si-H band after the treatment with HF, and also is a potential technique to detect pesticide residues in situ. Another possible application may be the detection of cell wall proteins in situ because it was observed for yeast cells that the spectra recorded following by SHINERS technique are different from references Raman spectra,

but are similar to those obtained from mannoproteins. As a conclusion, SHINERS may be an adequate technique to be world-wide used in a wide range of fields (26).

Cao YWC et al designed nanoparticles-based probes, labeled with Cy3 (Raman active dye), which are able to promote SERS for oligonucleotide targeting. Raman spectroscopy was founded to be an useful tool after elicit with Ag. Special chips were prepared with different DNA strands incorporated, the probe based on GNPs, oligonucleotides and Cy3 was added. Following the treatment with Ag, gray spots may be observed. Raman spectroscopy performed after Ag enhancement sustained the presence of Raman response, whilst no response was detected before Ag treatment. Cy3 dye was replaced from the nanoprobe with six other dyes and the detection method was performed for human immunodeficiency, hepatitis A and B, Ebola, variola viruses and Bacillus anthracis. The targets were mixed and were removed strategically to emphasize the selectivity of the method. The gray signals appeared were validated by Raman spectroscopy. RNA detection was also assessed and a semi-quantitative result was estimated. GNPs targeted with Raman active dye and specific oligonucleotide strain are veritable instruments in DNA or RNA detection, with advantages of great importance, such as the large variety of dyes, the wide range of probes and the possibility to obtain the ratio of specific intensity directly from the spectrum (27).

Starting from this, achievement, the team further investigated the possibility to develop protein probes. They designed two types of probes, with GNP core. The first probe designed, has an alkylthiol-capped oligoadenotides-biotin-Raman dye label, and it was used to assess screening of protein-small molecule interactions by using biotin, digoxigenin and dinitrophenyl as oligoadenotides. The chip was prepared containing specific antibodies. Following the enhancement with Ag, the

gray spots were visible and were further validated by Raman spectroscopy, indicating that the binding between probe and target took place. The experiment was performed also with only two probes to emphasize the selectivity. The second type of probe contains an antibody interposed between the GNP core and Raman dye and it was used to assess protein-protein interactions. The probes contained specific ubiquitin, mouse IgG and human protein C antibodies and the correspondent antigen was placed on slides. The experiment went further in the same manner as the first part, with the appearance of the gray spots and further confirmation via Raman spectroscopy. The method was founded to have a marked selectivity, it is also flexible, with no cross-reaction detected and it is also feasible as a protein detection technique. (28)

Schwartzberg AM et al developed GNP aggregates to be used as SERS substrate. The design of the surface is unique, due to the presence of sulfur species, confirmed by electron energy loss spectroscopy. UV-vis was used to characterize the particles. The spectrum exhibited two bands, the transverse plasmon band and the extended plasmon band, whether the spherical, non-aggregated particles have an unique transverse band. The interactions between the particles was revealed by TEM images. Furthermore, Rh6G was founded to increase SERS activity by 107 times. SERS signals have been registered in the presence of GNP aggregates for various amino acids and also for adenine and uridine. Clear distinct peaks never reported before have been registered, probably due to the unique properties of aggregates surface, or due to the maintenance of the pH at a value of 3, which is characteristic for GNP aggregates. (29)

In order to develop an effective method for bioimaging, Yin D et al developed Ag and SiO₂- based nanoparticles, labeled with sialic acid (SA). The nanoparticles were characterized by UV-vis. To evaluate the capacity to

differentiate normal from abnormal cells, normal liver cells (L-02) and human hepatocarcinoma cells (HepG2) were employed. After the nanoparticles were added, intense SERS signals may be evidenced in HepG2, whilst L-02 exhibited only weak signals. By incubation with non-labeled nanoparticles, no SERS signals may be evidenced. Glucose was also tested as an imprinting agent, but it was founded that it cannot differentiate normal from cancerous cells. Moreover, pre-blocking with glucose had no influence, whilst pre-blocking with SA led to the absence of response. By replacing SA with boronic acid, intense signals may be observed for both cell lines, suggesting the lack of selectivity. It may be concluded that the apparition of SERS signals, conditioned by specific binding, is related to the properties offered by SA. Furthermore, the method was performed for liver tissue, normal and abnormal. SA-targeted nanoparticles lead to intense SERS signals in damaged liver tissue. The results obtained from liver tissue are in well concordance with those obtained from cells experiments. Imprinted nanotags may represent key agents for improving bioimaging and cancer studies. (30)

Wan Xu et al proposed a design to incorporate lapatinib into human serum albumin nanoparticles as an effective agent in breast cancer with HER2 overexpression. The novel nanoparticles (LHNPs) have been characterized by Raman spectroscopy, which revealed that LHNPs lost the specific bands of lapatinib during incapsulation. The result is strengthen by XPS and XRS, leading to the conclusion that the incapsulation process took place conducting to a structure similar to human serum albumin. (31)

Fourier Transform Infrared Spectroscopy (FTIR) is also subjected to vibrational spectroscopy techniques, due to the excitation of oscillatory motions when the incident radiation has a frequency in the IR region (1-100 μm). The spectrum has nine bands: amide I-VII, A and B, which are characteristic for the repeated units of proteins and polypeptide. FTIR spectroscopy is useful to

determine the protein secondary structure with the great advantage that the amount required of sample is small. It is also appropriate for the study of dynamics and stability. The mention that the bands are superposed at the edges implies that the interpretation of the specific spectra should be performed with caution (32).

Sarmiento B et al proposed a delivery system for insulin, composed by alginate and chitosan (6:1), which was characterized by FTIR spectroscopy in order to assess chemical interactions. The spectra from chitosan, alginate and also from the alginate-chitosan complex has been reported, and revealed that an ionic interaction between carboxyl and amino groups of alginate and chitosan respectively occurred. The interactions recorded by adding the insulin payload were observed and it was concluded that are specific for protein entrapping (33).

FTIR has been used to evaluate silver NPs synthesis from papaya fruit extract. UV-vis spectra was registered to pursue the reduction process of Ag^+ , also macroscopic visible. FTIR performed before and after the bioreaction occurrence revealed that the reduction is probably due to the oxidation of C-O groups of polyos because the corresponding bands completely disappeared by adding Ag^+ ions and carbonyl bands were emphasized on the spectra (Jain D et al) (34).

Magnetite nanoparticles coated with PEG were used as a drug delivery system for methotrexate, which was immobilized on the surface of the NPs by an amine bond to improve the pharmacokinetics of the drug. FTIR was used to assert the success of the proposed structure. Moreover, UV-vis emphasized the cleavage process of the drug and the amount released. Considering the validation techniques performed, magnetite nanoparticles covered with PEG may be effectively used to improve methotrexate usage (35).

Several other nanoparticles and nanoscaffolds were characterized by FTIR:

collagen/nano-hydroxiapatite scaffolds (36), citric acid modified Fe_3O_4 and magnetic zeolitic imidazolate framework 8 (37), CdS QDs (38), CoFe_2O_4 , NiFe_2O_4 , MnFe_2O_4 (39).

Emission Spectroscopy

Photoluminescence and chemiluminescence are particular cases of emission. In first case, the emission appears after absorption and in the second case, the excited state is the result of a chemical reaction (2).

Photoluminescence spectroscopy

Both fluorescence and phosphorescence reunite photoluminescence spectroscopy field. In the first case, the energy emission is due to the transition in singlet excited state caused by photon absorption, in which the spin orientation of the excited electron is different from the ground electron. Phosphorescence appears as a consequence of energy emission from triplet excited state, in which the two spins have the same orientation. The spectra may be express in two different manners, emission and excitation spectra, according to the varied wavelength (2, 40).

Aslan K et al designed silver core nanoparticles with different silica shell thickness, which were bounded to various fluorophores- Rhodamine 800 (Rh800), Eu- [Tris (dibenzoylmethane) mono (5-amino phenanthroline) europium] (Eu-TDPA) and Alexa Fluor 647. Fluorescence spectra were realized and was compared to nanoparticles synthesized without the silver core. It was observed an increase by 8 fold and 20 fold in emission intensity for the nanoparticles labeled with Eu-TDPA and with Rh800 when compared to the control. Considering the decrease registered in the lifetime of the developed systems, it suggested the capacity of the nanocomposites to enhance particle sensing by 200 folds (41).

Fluorescence spectroscopy has been used for characterization of a variety of nanoparticles. Due to the hydrophobicity exhibited, curcumin was encapsulated into polyester nanoparticles. UV-vis and fluorescence spectra were performed and suggested that curcumin is located inside the nanosystem (Leung MHM et al) (42). CdS quantum dots (QDs) were used to form a nanosystem with chitosan and with a tripeptide labeled with chitosan (arginine-glycine-aspartic acid). Photoluminescent properties were evaluated by fluorescence spectroscopy, which revealed green fluorescence at 405 nm (Mansur AAP et al) (43).

A novel strategy for the assay of organophosphorus (OPP) has been reported (Zhang R et al). It is based on the inner filter effect (IFE) of GNPs, which quenches MnZnS QDs phosphorescence and also on the inhibition effect of OPP on acetylcholinesterase. In the presence of OPP, the production of acetylthiocholine is decreased and GNPs form aggregates which stimulate the restoration of the phosphorescence. Phosphorescence spectra suggested that the increase of GNPs concentration produces the decrease of phosphorescence MnZnS QDs emission. Moreover, the lifetime of QDs showed no noticeable change when AuNPs were added, suggesting that the complex is not relayed on hydrogen bonding and electrostatic forces but is due to the IFE of GNPs. The experimental data sustained the theoretical principles, which may lead to the assertion that an assay of OPP which implies phosphorescence spectroscopy is feasible (44).

Chemiluminescence (CL) is another emission spectroscopy technique. The excited state is due to a chemical reaction, which may occur directly from two reagents and cofactors forming excited state compounds. Bioluminescence is the result of biological or enzymatic reactions. Another mechanism of CL generation is the transfer of energy from the excited compound to fluorophores (2, 45).

Lee D et al have reported a novel method for in vivo imaging of hydrogen

peroxide. The technique involves nanoparticles with CL properties, made of peroxalate esters and fluorescent dyes. CL spectra was recorded from the peroxalate nanoparticles labeled with perylene, pentacene or rubrene in the presence of hydrogen peroxide. The spectra emphasized that the emission of light was recorded at a similar wavelength as the fluorescent emission of the dyes used. Moreover, the intensity of light exhibited a linear relation with the hydrogen peroxide concentration. The CL properties of the nanoparticles promises to be a key factor in in vivo imaging (46).

Due to the tremendous features of GNPS, CL have been studied by Cui H et al. GNPs react with $\text{KIO}_4\text{-NaOH-Na}_2\text{CO}_3$ and are able to elicit CL. The authors provided an insight into the correlation between the diameter of the nanoparticles and the CL intensity and it was observed that 68 nm GNP exhibited the strongest intensity. The spectra were also recorded and emphasized 3 different emission bands. The increase in CL intensity was observed by increasing the GNP concentration and by replacing the citrate ions of the surface with SCN^- . Further research is needed to establish the implication of GNPs in bioimaging. (47)

X-ray fluorescence spectroscopy (XRF) provides a qualitative and also a quantitative method by using measurements focused on specific wavelength and intensity of fluorescent emission energy, as a result of X-ray electromagnetic radiation (48). Even if the method is not destructive, the preparation process requires structural alternation of the analyte. A variant of XRF is micro-XRF, which offers the advantage to probe samples with irregular shape, by focusing on a small part of the sample (49).

In order to enquire into the destructions methods of tumor cells directly in the blood flow, Hossain M et al employed iron oxide and bismuth nanoparticles, which were functionalized with FA. The binding process was confirmed by

FTIR. HeLa and MG-63 cells were purchased, having overexpression of FA receptors (FAR) and an insignificant level respectively. The authors designed an *in vitro* system to simulate the blood flow in organism, in which HeLa cells treated with nanoparticles were added. The tube was engineered with a magnet on the internal wall, which is able to capture conjugated HeLa cells and by exposing to X-ray radiation, to destroy them. The experiment was performed also by replacing the PBS inside the tube with human blood cells containing HeLa and MG-63. XRF was performed after X-ray exposure and emphasized the selectivity of the system to capture HeLa cells, suggesting that the binding process took part because of the interaction between FA and FAR. Moreover, the affinity for FA-bismuth nanoparticles to bind to HeLa cells was asserted by XRF. The system proposed may have a well applicability on destroying cancerous cells in the blood flow by using targeted X-ray radiation and functionalized nanoparticles. (50)

CaCO₃ (51), arsenic acid-presenting iron oxide (52), selenium (53), Ag/SiO₂ (54) nanoparticles have also been used for various achievements and XRF was used as a method of validation.

Future Perspectives

Magnetic resonance spectroscopy (MRS) is a wide-available technique, used as a noninvasively, repetitively and painlessly diagnostic method, complementary to magnetic resonance imaging. It may indicate the concentration of a wide range of metabolites: N- acetyl aspartate, ATP, choline, creatine, phosphocreatine, lactate, applications that have tremendous importance especially in brain disorders (55, 56). By placing a nucleus in an uniform magnetic radiofrequency field (usually 10-100 MHz), it tend to register alignment and rotation movements and it also exhibits a transition process (57).

The resonance signal is the spectral line recorded after energy absorption, conducting to the spectrum registration (58).

The reaction between arsoncetic acid and dithiothreitol was evaluated by ¹H-MRS (52).

However, current data indicate that the potential of MRS has not been enquired in nanomedicine as it is other domains. Functionalized nanoparticles may be further studied regarding their ability to overcome MRS limitations and to improve the specificity of the presented technique.

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