Chapter 2

Materials and Methods

2.1. Introduction

This chapter primarily enlisted the source of chemicals and reagents used in the various experiments. The instrumental details for various experiments included in this thesis work have been briefly described.

2.2. Materials

The chemicals and reagents, which are used throughout my research work, are enlisted in Table 2.1 with their chemical formula and company name. The chemicals employed in this study were all of analytical grade and used without additional purification. Triple distilled water was used throughout the experimental studies. All glassware's were well cleaned with a mild HNO_3 solution before use and other contaminants were then removed by washing with triple-distilled water, alcohol and acetone solutions.

Table 2.1. List of chemicals, including formulas and company names

Name of the chemicals	Chemical formula	Company name
Sinapic acid	$C_{11}H_{12}O_5$	Sigma-Aldrich
Gallic acid	$C_7H_6O_5$	Sigma-Aldrich
Calf thymus DNA (ct-DNA)	---------	SRL
Ethidium bromide (EtBr)	$C_{21}H_{20}BrN_3$	SRL
Potassium Iodide	KI	Merck Millipore
Tris-buffer	$C_4H_{11}NO_3$	Merck Millipore
Ethyl alcohol	C_2H_5OH	Merck Millipore
MTT	$C_{18}H_{16}N_5S$	SRL
LDH-cell cytotoxicity assay kit	---------	HIMEDIA
DMSO	C_2H_6OS	Merck Millipore
PEG-4000	---------	Merck Millipore
Bromophenol blue	$C_{19}H_{10}Br_4O_5S$	Sigma-Aldrich
Nickel sulphate hexahydrate	$NiSO_4.6H_2O$	Sigma-Aldrich
Beta Casein	--------	Sigma-Aldrich

2.3. Characterization Methods

To learn more about physicochemical properties of the phenolic compounds (PCs) after interaction with the various macro molecules like, Deoxyribonucleic Acid (DNA), Bovine serum albumin (BSA), Human serum albumin (HSA) and encapsulation in milk β-Casein (β-CN) as well as after the formation of nano particle, it is important to use various analytical tools for their characterization. Microscopic, spectroscopic, and calorimetric studies are three main classes of instrumental methods for analysis.

- **Microscopic techniques:** In analytical science, this technique gives more information about visualization of single molecules, biological tissues, and nano materials. The three different types of microscopy are optical microscopy, electron microscopy, and scanning probe microscopy. Some the most common microscopic methods are transmission electron microscopy (TEM), scanning electron microscopy (SEM), and atomic force microscopy (AFM). The importance of this technique is to characterize morphology, surface structure and defects of various nanoparticles.
- **Spectroscopic techniques:** It is a powerful tool to deal with how the molecules respond to electromagnetic radiation and from that we know the interacting nature, size distribution, particle charges, etc., of the system. The most well-known spectroscopic methods are ultraviolet-visible (UV-Vis), fluorescence spectroscopy, fourier transform infrared (FTIR), atomic absorption spectroscopy (AAS), atomic emission spectroscopy, dynamic light scattering (DLS), zeta potential, powder X-ray diffraction (PXRD), X-ray photoelectron spectroscopy (XPS), nuclear magnetic resonance (NMR), Raman spectroscopy, and photoemission spectroscopy. This technique is used to study the interaction at the molecular level as well as identification or characterization of substances.
- **Calorimetric techniques:** Calorimetric techniques can be used to measure the thermal effects to establish a connection between temperature and specific physical properties of substances. The best-known calorimetric techniques are isothermal titration calorimetry (ITC) and differential scanning calorimetrie (DSC). This method is used to characterize the thermodynamic properties of the sample.

Several of the characterisation methods listed above was used for the implementation of the current work. The following sections provide a brief overview of each of the applied techniques.

2.3.1. Microscopic technique

Transmission electron microscopy (TEM)

TEM is a technique applied for analyzing the morphology of the specimen, i.e., surface structure, type of surface imperfection, i.e., defects, as well as crystal structure of the atom and size of the particle. The specimen composition is also determined through the TEM image. In order to scan a specimen in TEM, the specimen that is used must be exceedingly thin (particle dimensions are less than 100 nm in size or even down to atomic scale in some cases), because the electron beam must pass through this sample, and interact with the specimen to create the image [Wang et al., 2017]. Electrons transmitted through the specimen (called TEM grid, consisting of metal frame and carbon-based film) are focused by lenses and are projected on a phosphor screen to convert the electron image information to a visible form [Inkson, 2016]. For our experimental aims, the sample preparation for TEM measurements is discussed in the relevant chapter (cf. Chapter 4). The morphological nano particle was studied using transmission electron microscope (TEM) JEOL JEM 2100 HR with EELS.

2.3.2. Spectroscopic techniques

2.3.2.1. Dynamic light scattering (DLS)

A particularly well-defined method for determining the particle size distributions in colloidal suspensions and emulsions is dynamic light scattering. DLS is frequently used to characterize the particle sizes (hydrodynamic radius) of the nano carriers (unloaded or loaded with PCs), suspensions of individual nanoparticles, or aggregates of nanoparticles for a variety of analytical applications, such as evaluating dispersion techniques or verifying product specifications. DLS is used to estimate the Brownian motion using Stokes-Einstein equation of individual particles in liquids for the justification particle size analysis [Babick, 2020]. The measured particle size in DLS is always higher with respect to TEM. Because DLS is measured in the solvated state whereas, TEM is performed in the dry state. Another reason for this deviation is that DLS is an intensity-based measurement and emphasize on the larger particle size whereas, TEM is a number based particle size measurement that show stronger emphasis on the smallest components in the size distribution [Souza et al., 2016]. DLS measurement is discussed in the relevant chapter (cf. Chapter 5).

2.3.2.2. Fourier transform infrared (FTIR)

FTIR spectroscopy is a vibrational spectroscopic method that can be used to optically investigate the chemical alterations (dipole moments of a molecule) of a molecule when it is connected with the other molecule. The main principle of FTIR is that when the covalent bonds of a molecule selectively absorb radiation of specific wavelengths, causing the vibrational energy in the bond to shift. The type of vibration (stretching or bending) caused by infrared light depends on the atoms in the bond. The transmittance pattern varies for molecules because different bonds and functional groups absorb different frequencies (The opposite of absorption is transmittance and frequency range is 4000 to 400 cm^{-1}). Wavenumber (cm^{-1}) is plotted on the X-axis and transmittance is plotted on the Y-axis to represent the spectrum (Wavenumber is 1/wavelength and represents the energy of the molecular bonds' vibrational motion). FTIR spectroscopy is utilised various field in science like, organic synthesis, petrochemical engineering, polymer science, pharmaceutical business, and food analysis [Sindhu et al., 2015]. This measurement is discussed in the relevant chapter (cf. Chapter 4). The analysis of functional groups was performed using a Perkin-Elmer L120-00A Fourier Transformed Infrared Spectrometer with sample as KBr pellets.

2.3.2.3. Powder X-ray diffraction (PXRD)

PXRD is an analytical technique mostly used for identifying the phase of a crystalline material and providing information on unit cell dimensions. The studied material is finely powdered and homogenised before determining the average bulk composition. The diffraction pattern is made up of a series of sharp Bragg reflections that match to the distinct d-spacing of the material. Before the crystallographic investigation, the powder sample was placed on a rectangular glass holder that is being presented in front of the X-ray [Thomas et al., 2006]. The broadening of peaks in the PXRD pattern is predicted by instrumental parameters, commonly attributed to crystallite size effects. The Scherrer broadening of lines is caused by small crystallites [West, 2022]. Anisotropic crystallites are responsible for any non-uniform widening of lines. For the determination of a nano particulate material, the Scherrer broadening is now used extensively to estimate crystallite size. PXRD is also used for the identification of unknown crystalline materials like minerals, inorganic compounds as well as geology, environmental science, material science, engineering and biology [Ding

et al., 2001]. This measurement is discussed in the relevant chapter (cf. Chapter 4). A powder X-ray diffractometer Rigaku Smart Lab Automatic High Resolution Multipurpose PC Controlled X-Ray Diffractometer System was used for the X-ray diffraction pattern of the nanoparticles.

2.3.2.4. X-ray photoelectron spectroscopy (XPS)

One of the most extensively utilized surface-sensitive electron emission spectroscopic techniques is X-ray photoelectron spectroscopy. Atomic core electrons are held in their orbitals by their distinctive binding energies. The binding energies are influenced by the properties of nearby atoms, orbital types, and atom types. XPS measures the binding energies of electrons released from the sample surface. For this the sample surface is bombarded with soft x-ray photons, typically 1486 eV from an Al-anode [Stevie et al., 2020]. The photon penetrates in to the sample surface to a depth of around 1 μm. When a photon collides with a sample atom, the energy of the photon ionises core electrons, causing them to be expelled from the atoms. The mean free path of the expelled electrons within the bulk of the sample is very short due to interaction with the surrounding atoms. These electrons will be subjected to ultrahigh vacuum (UHV) conditions within the spectrometer to avoid scattering by gas molecules and to limit surface contamination during the examination. The kinetic energy of the expelled electrons is measured by an electron energy analyzer (spectrometer) [Stevie et al., 2020]. This major characteristic of XPS makes it as powerful analytical techniques for its surface sensitivity and the capability to reveal chemical state information from the components of the sample [Stevie et al., 2020]. XPS measurement is very useful tools for the researchers to understand the surface phenomenon of the materials, as the surface phenomenon depends on the adhesion, corrosion, charge transfer, surface wettability, and catalysis etc. XPS analyze the surface of almost all materials, including semiconductors, polymers, textiles, and soil but it cannot detect the hydrogen and helium [Briggs, 2003]. This measurement is discussed in the relevant chapter (cf. Chapter 4).

2.3.2.5. UV-Vis spectroscopy

The fundamentals of spectroscopy is based on the interaction of light with matter. In case of UV-Vis spectroscopy, chemical compounds absorbs the ultraviolet light or visible light

(190-800 nm; UV region: 190-400 nm and visible region: 400-800 nm) and the electrons present in the compounds undergo excitation [Moldoveanu et al., 2017]. This causes them to jump from a ground state to an excited state, which results in the production of distinct absorption maxima in the spectra. Generally, organic compounds shows the transition as n-π* and π-π*. PCs also exhibits the transition for n-π* (range 300-550 nm) and π-π* (range 200-280 nm), resulting the absorption maxima [Valeur et al., 2012; Lakowicz, 2006]. Another frequent application of UV-Vis spectroscopy is in Job's approach, which yields the host-guest inclusion complex or metal complex's binding stoichiometry [Valeur et al., 2012]. The other applications are the drug release behavior, encapsulation efficacy, and bioaccessibility etc.

This spectroscopic study will also help to investigate how DNA interacts with PCs. The thermal melting experiments using UV-Vis spectroscopy also give some idea about the types of binding (intercalation/groove/electrostatic) between DNA and PCs. As the static fluorescence quenching involves the ground-state complexation, hence the static quenching can also be studied by UV-Vis spectroscopy but the dynamic quenching cannot be determined by UV-Vis spectroscopy as it is an excited-state phenomenon [Lakowicz, 2006]. UV-vis spectroscopic measurement was performed on a Hitachi U-2910 UV-spectrophotometer at room temperature using a cuvette of 1.0 cm x 1.0 cm path length.

2.3.2.6. Steady-state fluorescence spectroscopy

Fluorescence is the emission of light by a material that has absorbed light or other electromagnetic energy. It is a type of photoluminescence (photo= light; luminescence = the emission of light). In brief, when light of an appropriate wavelength is absorbed by a molecule (i.e., excitation), the electronic state of the molecule changes from the ground state (singlet state, denoted as S_0) to one of many vibrational levels in one of the excited electronic states (S_n with $n > 0$) of the same multiplicity (spin) of the ground state, usually a singlet state and finally return to the ground state. This type of relaxation processes is known as fluorescence [Lakowicz, 2006; Naveenraj et al., 2013]. Fluorescence spectrophotometry is one of the most common techniques in the fields of biochemistry and molecular biophysics. The approach has gained popularity because to its high sensitivity towards changes in the structural and dynamic characteristics of biomolecules and bimolecular complexes, thus widely employed to investigate the nature of the interaction.

Fluorescence quenching refers to a physicochemical process that reduces the intensity of fluorescent molecules. Generally, BSA interacts with bioactive molecules by quenching the intrinsic fluorescence of the protein, i.e., the bioactive molecules act as a quencher in this interaction process. A variety of molecular interactions can result in quenching. Some examples of common chemical quenchers are O_2, I^-, Cs^+ and acrylamide [Lakowicz, 2006]. These include molecular rearrangements, excited-state reactions, energy transfer, ground-state complex formation, and collisional quenching interaction. Generally, quenching is divided into three categories such as static quenching, dynamic quenching, and combined quenching (static-dynamic both) [Lakowicz, 2006]. Steady state emission spectra measurement was obtained with a Cary Eclipse fluorescence spectrophotometer (model G9800A) using 1.0 cm quartz cells.

2.3.2.6.1. Static quenching

The static quenching occurs by the formation of the ground state complex between the fluorophores and quencher molecule, i.e. the formed complex is non-fluorescent in nature and the complex is formed before excitation. In this case, the dependence of the fluorescence intensity on quencher concentration may be easily derived by consideration of the association constant for complex formation. This constant is given by the following equation.

$$\mathbf{K_S = \frac{[F-Q]}{[F][Q]}} \qquad (2.1)$$

Where, [F-Q] is the concentration of the fluorophore-quencher complex, [F] is the the concentration of uncomplexed fluorophores, and [Q] is the quencher concentration. If the complex is nonfluoroescent, then the fraction of remaining fluorescence is F/F_0 and this is equivalent to the fraction of total fluorophores that are not complexed. As the total concentration of fluorophore is

$$\mathbf{[F]_0 = [F] + [F-Q]} \qquad (2.2)$$

Substituting the valve of [F-Q] into the equation (2.1.) yields

$$\mathbf{\frac{[F]_0}{F} = 1 + K_S[Q]} \qquad (2.3)$$

The static quenching removes a fraction of available fluorophores from emitting without affecting the remaining uncomplexed fluorophores. The fraction of uncomplexed

fluorophores is unaffected, and hence lifetime is τ_0. Therefore, for static quenching $\tau_0= \tau$ and for dynamic quenching $F_0/F= \tau_0/\tau$.

2.3.2.6.2. Dynamic quenching

Dynamic or collisional quenching occurs by interaction of an excited state fluorophores with the quencher that results in radiation less deactivation of the fluorophores to the ground state. Hence, the fluorophores and quencher must diffuse together within the timescale determined by the excited state lifetime τ_0. The dynamic quenching is described by Stern-Volmer equation. There are some ways to differentiate between static and dynamic quenching. The first method is fluorescence lifetime studies (discussed briefly in the next section). In the case of dynamic quenching, the additional deactivation pathway reduces the observed lifetime of the fluorescence. Whereas for static quenching, the lifetime does not change since it is ground state phenomenon [Lakowicz, 2006]. The other method to discriminate between these two quenching process, one may carefully analyse the impact of temperature on quenching efficiency. In dynamic quenching process the quenching efficiency is expected to increase with increase in temperature due to larger diffusion coefficients at higher temperature. In contrast, for static quenching, with increasing temperature the stability of the complexes decreases hence reduce the static quenching efficiency [Lakowicz, 2006]. The phenomenon of fluorescence quenching can be well-described by the Stern-Volmer equation given below [Lakowicz, 2006]:

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] = 1 + K_D[Q] \qquad (2.4)$$

where, F_0 and F represent the fluorescence intensities of the fluorophore in the absence and presence of a different concentration of quencher ([Q]), respectively. k_q is the bimolecular quenching constant, τ_0 is the fluorophore lifetime in the absence of a quencher, and K_{SV} or K_D is the Stern-Volmer quenching constant.

However, it is important to recognize that there are many processes where both dynamic and static quenching processes occur simultaneously. When, both the process is occurred in a sample, the dynamic portion of the observed quenching be determined through lifetime measurements (discussed later) but if the life time measurements are not available then the portion of static and dynamic quenching can be obtain through a graphical separation of K_S and K_D [Lakowicz, 2006].

$$\frac{F_0}{F} = 1 + (K_D + K_S)[Q] + K_D K_S [Q]^2 \quad (2.5)$$

$$\frac{F_0}{F} = 1 + K_{app}[Q] \quad (2.6)$$

where,

$$K_{app} = \left[\frac{F_0}{F} - 1\right]\frac{1}{[Q]} = (K_D + K_S) + K_D K_S [Q] \quad (2.7)$$

where, F_0 and F represent the fluorescence intensities of the fluorophore in the absence and presence of a different concentration of quencher ([Q]), K_S and K_D are static and dynamic quenching constants, K_{app} is called apparent quenching constant. A plot of K_{app} versus [Q] yields a straight line with an intercept of $K_S + K_D$ and slope of K_SK_D from this intercept and slope value we can determine the K_S and K_D value separately.

The fluorescence quenching interaction of some biological macromolecule with PCs gives the valuable information about the binding constant, thermodynamic parameters, and probable binding site as well as the nature of binding (groove/intercalation) using ethidium bromide and potassium iodide etc. The above mentioned experimental methods are discussed in the relevant chapters (Chapter 3B, 3A, 5, and 6).

2.3.2.7. Time-resolved fluorescence studies

Time-resolved fluorescence spectroscopy investigates how the fluorescence of a sample varies over time when it is subjected to UV, visible, or near-IR light. The decay time of fluorophores can be measured over a wide time range: from picoseconds to milliseconds and beyond. This average time duration of a fluorophore in its excited state is known as fluorescence lifetime (τ). Fluorescence lifetime (τ) is an intrinsic property of a fluorophore. The lifetime of the fluorophore is influenced by the microenvironments the fluorophore, hence fluorescence lifetime (τ) does not depend on fluorophore concentration, sample thickness, absorption by the sample, method of measurement, fluorescence intensity, photo-bleaching, and/or excitation intensity. Fluorescence lifetime (τ) is influenced by the local properties of the fluorophore microenvironments like temperature, polarity, and the presence of fluorescence quenchers as well as sensitive to internal factors that are dependent on fluorophore structure [Berezin et al., 2010].

The following Stern-Volmer equation is utilized to evaluate the dynamic quenching [Lakowicz, 2006]:

$$\frac{\langle\tau_0\rangle}{\langle\tau\rangle} = 1 + K_D[Q] \qquad (2.8)$$

where, $<\tau_0>$ and $<\tau>$ are the average lifetime of fluorophores in the absence and presence of the quencher, respectively, and K_D is the dynamic quenching constant. For static quenching, the fluorophore-quencher complex is nonfluorescent and thus, only the free fluorophore is responsible for the resulted fluorescence. Since the free fluorophore remained unperturbed, its lifetime remains at τ_0, thus making the overall fraction $\tau_0/\tau = 1$. On the other hand, for dynamic quenching, τ_0/τ is equal to or close to F_0/F.

Time-resolved fluorescence measurements can also be used to characterise how the host molecule interacts with the fluorescent guest molecule. Fluorescence lifetimes were measured by the method of Time Correlated Single-Photon Counting (TCSPC) using a HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer.

2.3.2.8. Steady-state fluorescence anisotropy

Steady-state fluorescence anisotropy measures the difference between rotational mobility of bound and free molecule. It is a commonly used technique for examining protein-protein, protein-DNA, ligand-DNA and protein-ligand interactions [Thompson, 2009]. For measuring fluorescence anisotropy, incident light is vertically polarised so that the excitation light's electric vector is parallel to the z-axis [Lakowicz, 2006]. The fluorescence intensity of the components is measured through polarization in the vertical direction (parallel) or the horizontal direction (perpendicular). The fluorescence anisotropy (r) can be calculated by the following equation.

$$r = \frac{(I_{VV} - G \times I_{VH})}{(I_{VV} + 2G \times I_{VH})} \qquad (2.9)$$

$$G = \frac{I_{HV}}{I_{HH}} \qquad (2.10)$$

Here, I is the fluorescence emission intensity and the suffix VV denotes both the excitation and emission polarizers to be vertically aligned and VH indicates a vertically aligned excitation polarizer and horizontally aligned emission polarizer and so on. G is the correction factor for the polarization dependence of the spectrofluorometer [Lundblad et

al., 1996; Valeur, 2001]. Fluorescence anisotropy depends on the size and shape of the fluorophore, as well as its local environments. For a spherical molecule anisotropy decay is expected due to a single rotational correlation time. The multi-exponential anisotropy decay is shown for non-spherical fluorophores or proteins. Larger macromolecules with a fluorophore are shown to be the complex anisotropy decay due to its internal flexibility. Anisotropy decays can also be affected by changing the concentration of the fluorophore, as they transfer their energy through resonance between them. This depolarization is called homotransfer [Lakowicz, 2006]. This measurement is discussed in the relevant chapter (cf. Chapter 3A and 6). Steady-state fluorescence anisotropy (r) measurements were performed on Cary Eclipse fluorescence spectrophotometer (G9800A) equipped with a 1.0 cm path-length of rectangular quartz cell.

2.3.2.9. Circular Dichroism (CD) studies

Circular dichroism is the difference between the absorption of left (E_L) and right (E_R) handed circularly polarised light as a function of frequency that is commonly observed in chiral compounds [Berova et al., 2000; Hammes, 2005]. Circular dichroism is also known as dichroism for spin angular momentum because left-hand circular (E_L) and right-hand circular (E_R) polarised light represent two possible spin angular momentum states for a photon [Fournier, 2013]. Chirality in molecules can be observed due to their intrinsic structure or if they are placed in an asymmetric environment [Hammes, 2005]. In CD spectroscopy, the incident unpolarised light is first transformed into plane polarised light, and then it is divided into its E_L and E_R circularly polarised components by a photo-elastic modulator, which is made up of a piezoelectric element attached to a block of fused silica and functions as a dynamic quarter wave plate [Berova et al., 2000]. According to the substance's chirality, one component is absorbed more than the other, producing an elliptically polarized transmitted light [Berova et al., 2000; Kelly et al., 2000]. In CD experiments, the difference in absorption ($\Delta A = A_L - A_R$) or ellipticity (θ) as a function of wavelength is measured, and depending on the degree to which the E_L and E_R components are absorbed, negative or positive signals are seen; for achiral substances, $\Delta A = 0$, as they equally absorb both components. The relationship between ΔA and θ is $\theta = 32.98\ \Delta A$. Asymmetric carbons in biomolecules make them chiral, which makes CD valuable for examining their structures. The helicity and base stacking of various DNA structures affects their chirality which results in differences in the CD signals. This measurement is discussed

in the relevant chapter (cf. Chapter 3B and 6). CD measurements were performed on a JASCO J-815 spectrometer using a rectangular quartz cuvette of path length 1 cm at room temperature.

2.3.3. Calorimetric techniques

Isothermal titration calorimetry (ITC)

ITC is a common technique used to determine the thermodynamic parameters of interactions in solution. ITC often used to study the binding interaction between small molecules (like phenolic compounds) with larger macromolecules (proteins, DNA etc.). It also provide a valuable information about thermodynamic parameters like enthalpy change (ΔH), entropy change (ΔS), free energy change (ΔG) as well as binding affinity (K_a) and binding stoichiometry (n) for a host-guest interaction in solution [Pierce et al., 1999]. This measurement is discussed in the relevant chapter (cf. Chapter 4). The change in thermodynamic parameters was examined using Malvern MICROCAL PEAQ-isothermal titration calorimetric (ITC) instrument.

2.4. Instruments used

The following instruments have been used for determination of various interaction processes throughout the experiments.

Transmission electron microscope (TEM) was performed using JEOL JEM 2100 HR with EELS instruments.

Fourier Transformed Infrared Spectrometer was used to determine the functional groups using Perkin-Elmer L120-00A instruments.

A powder X-ray diffractometer Rigaku Smart Lab Automatic High Resolution Multipurpose PC Controlled X-Ray Diffractometer System was used for the X-ray diffraction pattern of the nanoparticles.

UV-vis spectroscopic measurements were performed on a Hitachi U-2910 UV-spectrophotometer.

Steady state emission spectra measurement was obtained with a Cary Eclipse fluorescence spectrophotometer (model G9800A).

Fluorescence lifetimes were measured by the method of Time Correlated Single-Photon

Counting (TCSPC) using a HORIBA Jobin Yvon Fluorocube-01-NL fluorescence lifetime spectrometer.

Steady-state fluorescence anisotropy (r) measurements were performed on Cary Eclipse fluorescence spectrophotometer (model G9800A).

CD measurements were performed on a JASCO J-815 spectrometer instruments.

Isothermal titration calorimetric (ITC) were performed using Malvern MICROCAL PEAQ instrument.

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