2.1 Chronic myeloid leukaemia (CML)

CML is a myelo proliferative disorder with an incidence of 2.0 per 1,00,000 adults, and mortality rate of 0.3 per 1,00,000 adults annually and accounting for about 20 % of newly diagnosed cases of leukaemia in adults [Jabbour et al., 2012, Siegel et al., 2016]. The molecular characteristic of CML is a shortened and truncated version of the Philadelphia chromosome (chromosome 22) present in more than 90 % of patients [Rowley, 1973]. This abnormality leads to the swapping of DNA between chromosomes 9 and 22. The subsequent fusion of the Abelson murine leukaemia (ABL) gene on chromosome 9 with the breakpoint cluster region (BCR) gene on chromosome 22, results in the BCR-ABL oncogene [Quintas and Cortes, 2009]. This ultimately leads to an unregulated activity and triggers a cascade of events that affect the cellular proliferation and control of apoptosis.

The onset of CML comprises of three distinct phases: chronic phase, accelerated phase and blast crisis. CML is usually diagnosed in chronic phase (80-90 % of newly diagnosed cases), which is relatively long-lasting and is characterized by accumulation of blast cells (myeloid progenitors) in the blood, bone marrow and spleen [Santos et al., 2011]. The most common symptoms observed in the chronic phase are anaemia, splenomegaly, general pain, illness, fatigue and weight loss [Jabbour and Kanterjian, 2014]. The disease may progress into an accelerated phase over the years due to the rapid expansion of circulating blast cells. Ultimately, CML may progress to blast crisis when more than 30 % immature blast cells, myeloid lineage or lymphoid lineage, are there in blood, bone marrow or spleen, resulting in a sharp rise in the protein levels and BCR-ABL mRNA [Baccarani et al., 2015]. Blast crisis usually leads to a median survival of 3 months without proper treatment due to poor prognosis and worsening

symptoms like fever, infections and bleeding, metastasis, organ failure and finally leading to death [Diger, 2012]. The triphasic model of CML progression can be explained by the genomic alterations due to the genetic instability of blast cells, activation of pathways that block myeloid differentiation and inactivation of tumor suppressor genes. These molecular events extend LSC (leukemic stem cells) replicative lifespan [Perrotti et al., 2010] and altogether they may serve as key targets for curing CML [Chen et al., 2010].

2.1.1 Diagnostics and therapeutics

The clinical symptoms of CML are often not specific, and most of the patients who are diagnosed with CML are asymptomatic (~ 40 % in the US alone). The diagnosis of CML will usually be suspected from the elevated WBC (white blood cell) count or when splenomegaly is detected during the routine blood test or general physical examination [Jabbour and Kanterjian, 2014]. Biopsy of bone marrow is mandatory after performing the hematological characterization in order to confirm the diagnosis. The current state of diagnosis of CML consists of molecular biology screening, cytogenetic testing like FISH or RT-qPCR and imaging tests like computed tomography or ultrasound examination of the spleen [Baccarani et al., 2015].

The treatment of CML mainly depends on the genetic profile, disease state, health and age of the patient and prior history of treatment. Most of the CML patients are treated with chemotherapy, while a few may also undergo radiation therapy, stem cell transplantation, targeted therapy or combined strategies of these treatments [Ou et al., 2008]. Until the early 1990s, the best temporary disease control therapy for chronic phase CML was administering nonspecific agents such as busulfan, hydroxyurea, and IFN-α (interferon-alfa). Although IFN-α is competent in reducing the proportion of the Ph chromosome, it is associated with multiple side effects [Bonifazi et al., 2001].

Radiation therapy also known as radiotherapy involves the use of high doses of high energy radiation such as gamma rays, x-rays and neutrons to kill cancer cells, and shrink tumors. The radioactive substances transfer its energy into highly energetic electrons which ionize the DNA. This ionization of DNA inhibits cell division or causing cell death. Cancerous cells are more vulnerable to the ionization due to the fact that they are more unstable in all aspects. One of the major disadvantages of radiation therapy is that it is impossible to target only the tumor cells, without causing any harm to the surrounding healthy cells. Stem cell transplantation became the choice of treatment from the early 1990s onwards, resulting in curative interventions. The problems associated with stem cell transplantation are a high rate of morbidity and mortality, requires an appropriate stem cell donor and also relatively young patients [Hansen et al., 1998]. With the design and development of the tyrosine kinase inhibitors (TKI), the CML treatment strategies and outcomes boosted, and TKI therapy is considered as the standard first line treatment for patients with newly diagnosed CML [Giles et al., 2005]. The TKI recommended in the treatment of CML are shown in Table 2.1.

Table 2.1 Tyrosine kinase inhibitors (TKI) in the CML treatment [Mathisen et al., 2014, Leach, 2019]

TKI	Generation	Indications	FDA Approved (month/ year)
Imatinib	I	First line therapy for chronic phase CML.	5/2001
		After the failure of IFN-α therapy in chronic, advanced or blast crisis phase CML.	4/2003
Dasatinib	II	For patients resistant or intolerant to prior therapy (chronic, advanced, and blast crisis phases).	6/2006
		First line therapy for chronic phase CML.	10/2010
Nilotinib	II	For patients resistant or intolerant to prior therapy (chronic and advanced phase CML).	10/2007
		First line therapy in chronic phase CML.	6/2010
Bosutinib	II/III	For patients resistant or intolerant to prior therapy (chronic and advanced phase CML).	9/2012
Ponatinib	III	As second-line therapy or beyond when no other TKI is indicated.	3/2013

Although Imatinib exhibits the ability to induce the complete haematological response in chronic phase CML patients, its monotherapy is not effective in most of the patients due to the incomplete eradication of BCR-ABL expressing leukemic cells [Lin et al., 2014]. The most important concern with the Imatinib is the development of resistance or intolerance in patients. Besides Imatinib, Dasatinib (DSB) has indication for first line therapy in CML. The selection of the most suitable TKI is based on patient age, comorbidities, drug cost, blood counts at diagnosis, and drug toxicity profile which altogether ensures that the treatment is well tolerated, target-oriented, adequate, and with minimal impact on patients' quality of life [Baccarani et al., 2014]. DSB is a more potent inhibitor than Imatinib and has shown the ability to induce the complete hematologic response in newly diagnosed patients. However, its impact on long term overall survival remains undetermined [Tokarski et al., 2006].

There is a strong need for a second generation TKI due to the fact that about 30 % of CML patients will develop BCR-ABL dependent or independent resistance to the first generation TKI therapy [Fausel, 2007]. Over expression of the BCR-ABL oncogene and BCR-ABL kinase mutations are the prime reasons for the development of resistance [Gorre, 2001]. Second generation TKI therapy is has been proved to be highly effective against Imatinib resistance in CML patients [Jabbour et al., 2011]. A recurrent cause of chemotherapy failure may arise due to Imatinib exposure which may ultimately leads to a multidrug resistance (MDR) phenomenon [Burger et al., 2005].

2.1.2 Current therapeutics

With the advent of the second generation TKI, fatal cancer has converted into a manageable chronic disease in CML patients and the survival rate (8-year) is recorded at about 90 % since 2001. Also, the life expectancy of CML patients is improved and reached close to that observed for the general population [Björkholm et al., 2011]. Though most of the CML patients respond to chemotherapy very well, still the clinicians are facing quite a few challenges like the recurrence of leukaemia in some patients [Mahon, 2012] and the safe time at which a successful treatment shall be discontinued [Carella et al., 2013]. Continuing the medication permanently poses a lot of problems like non-compliance with treatment, fertility and pregnancy issues in the women, economic burden and development of resistance. Precision medicine seems to be the way forward for many types of cancers in which a more specific chemotherapy strategy is suggested to individual patients after performing an accurate biochemical and genetic profiling will greatly improve the outcome. These therapeutic approaches and the personalization of CML management shall be made possible with the help of more sensitive and easy diagnostic strategies in conjunction with the novel molecules targeting specific subtypes of CML causing aberrations. By combining the benefits of

nanotechnology, it is feasible to put together imaging, targeting and therapeutic agents in a single vehicle (nanotheranostics), indulging in real-time monitoring of disease state, minimization of side-effects, improving target specificity, reduction of therapeutic doses and repeated administrations. This strategy when exercised at an early stage of the disease will mutually benefit the patients and the economics of healthcare [Pedrosa et al., 2015, Vinhas et al., 2015].

2.2 Nanomedicine

Conventional approaches for the management of cancer depend on the time-consuming and complex centralized diagnostic platforms that are set for the broad spectrum of malignancies and patients [Friedman et al., 2015]. These platforms have been established to identify suitable biomarkers and profiles, for which standard chemotherapeutics protocols are designed [Zaimy et al., 2017]. This approach paved the grounds for the improvement of precision oncology, in which the cancer cells in the individual patients receives a tailored treatment.

Nanoparticles are synthesized from diverse materials in the range of 1-100 nm. Because of its versatile functional and structural properties, they offer the opportunity for unfolding a specific, sensitive and rapid diagnostics, decentralized evaluation and/or ambulatory follow-up [Vinhas et al., 2017]. As compared to their bulk materials, nanoparticles possess unique and improved physico-chemical properties due to their substantial reactive surface area and quantum size effect. They also possess unique electrical, magnetic, optical and catalytic properties which make them more distinctive and relevant than large size materials [Tatar et al., 2016, Nikalje et al., 2015]. All these unique properties of nanoparticles are influenced by their size and shape, hence much diligence has to be paid towards controlling their size and shape. There is a wide variety of nanoparticles like liposomes, dendrimers, quantum dots, polymers, metal (e.g.

magnetic, gold, silver, iron oxide) and carbon [Mendes et al., 2016]. The extensive applications of nanoparticles in the field of biomedicine to deliver the drugs are attributable for their theranostic approaches [Tinkle et al., 2014]. As nanoparticles have large surface area, their surfaces can be accessible for further alteration with hydrophilic, hydrophobic, cationic, anionic or any neutral moieties to the surrounding environment so that their applications can be drawn out to further extent. This flexibility is of particular interest for theranostics approaches, offering the possibility of simultaneous cancer detection and therapy [Pedrosa et al., 2015]. Among the plethora of different nanocarriers applied to cancer theranostics, the present research will be focused on gold nanoparticles (GNPs) and its advantages in cancer management.

2.3 Focus on GNPs

GNPs are available in different shapes and sizes, and their unique properties make them suitable for various applications as shown in Figure 2.1. GNP's electronic properties facilitate their use as sensitizers in radiotherapy with 3-6 folds improved potential when compared to the substances usually used in clinical trials (e.g., gadolinium complexes) [Hainfeld et al., 2013]. GNP's optical properties facilitate them to induce hyperthermia at the tumor site upon irradiation by NIR light and show enhanced potential in photothermal therapy [Kodiha et al., 2014].

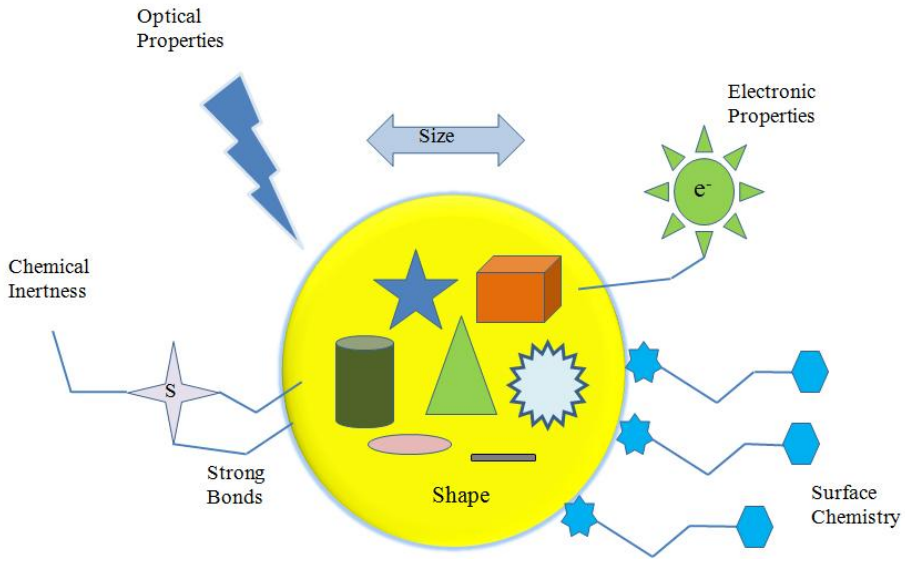


Figure 2.1 Graphical representation of main properties of gold nanoparticles [Adena et al., 2018]

GNPs turned out to be excellent drug nanocarriers for anti-cancer drugs as they can effectively carry a high therapeutic payload and selectively release the chemotherapeutic drug at the tumor micro environment [Kim et al., 2013]. They are selectively delivered at the tumor micro environment by active or passive targeting methods. The former is accomplished by conjugation with ligands having an affinity for the receptors expressed on malignant cells or its microenvironment. Various ligands used for this purpose include antibodies, oligosaccharides, organic molecules, and peptides. GNPs adopt dual way approach to killing cancer cells by delivering the anti-cancer drugs effectively at the tumor site and exert photoablation therapy. The latter is accomplished by EPR (enhanced permeation and retention) effect in which GNPs reaches the interstitial space of the tumor through the leaky vasculatures, and the impaired lymphatic drainage constrains the clearance of the GNPs [Nichols and Bae., 2014]. GNPs tend to accumulate and interact with the cancer cells due to their distinctive enhanced permeability and retention property [Choi et al., 2015].

GNPs have a variety of applicability over conventional contrast agents as gold has a higher atomic number and high absorption coefficient because of its electron density, so it increases computed tomography contrast further and is used as molecular probes in X-ray computed tomography imaging [Amjadi and Farzampour, 2014]. GNPs surfaces are readily accessible for adaptation with specific biomarkers or targeting molecules and are utilized in biomedical prospects [Guo et al., 2014, Lan et al., 2013]. They are widely used as contrast agents in molecular imaging such as MRI, CT, PET, ultrasound and optical imaging [Qie et al., 2015, Tiedemann et al., 2014]. GNPs can get tunable absorption due to their unique anisotropic geometry and can be potentially used in the fields of photothermal therapy and biosensing [Pal et al., 2013]. Due to its surface plasmon resonance (SPR), GNPs are widely used in tumor imaging, photoablation therapy, drug delivery and immune chromatographic detection of pathogens [Yang et al., 2014]. GNPs have a wide variety of applications in *in-vivo* imaging due to its SPR and scattering properties [Rossi et al., 2016]. Surface modification of GNPs with a thermolabile polymer is utilized as a drug carrier which upon interaction with near infrared radiation releases their effectors. Because of their distinctive electronic and optical properties, GNPs have been extensively used in color indicating probes for the sensing of various analytes [Murawala et al., 2014].

2.4 Synthetic strategies of preparing GNPs

GNPs of different nanoshapes are reported based on the synthesis method employed and the experimental conditions involved, including gold nanospheres, nanoshells, nanocages, nanorods, nanoclusters, nanoboxes, nanocrystals, nanocubes, and nanostars [Khan et al., 2013]. However, when compared to other nanoshapes gold nanospheres, nanoshells, nanocages, and nanorods are widely investigated for various

theranostic applications. Various synthesis methods, including chemical, physical and green methods have so far been introduced.

2.4.1 Chemical methods

In chemical method, the synthesis of GNPs involves the reduction by using reducing agents like citrate acid, borohydrides, oxalic acid, formaldehyde, hydrogen peroxide, acetylene, and stabilization by using stabilizing agents like oxygen, phosphorus, and nitrogen based ligands, polymers and surfactants. Turkevich method is the well-known, and widely used method for the synthesis of GNPs in which tetrachloroauric acid in water is boiled, and the reducing agent trisodium citrate dehydrate is rapidly added into it with vigorous stirring. The color of the resulting solution changes from light yellow to wine red after a few minutes. In this method, there is no need of adding a stabilizing agent as citrate ions play both the role of reducing and stabilizing agents [Turkevich et al., 1951]. Brust-Schiffrin method is an easy and simple method for the stable GNPs synthesis with controlled size and good colloidal dispersity. In this method, tetraoctyl ammonium bromide is used as a phase transfer agent which transfers the aqueous $AuCl_4$ solution to a toluene phase. The resultant is reduced by using sodium borohydride in the presence of dodecanethiol. The thiol stabilized GNPs results from a color change of orange to deep brown [Brust et al., 1994]. Other nanoshapes can be synthesized besides growing of GNPs of spherical shape.

In general, non-spherical GNPs have a tendency to aggregate. High curvature of GNPs and the nature of ligands on their surface are the main factors that influence the flocculation. Smaller GNPs due to a less number of ligands capping while larger GNPs due to inter-particular interaction via weak hydrogen bonds shows a low affinity towards flocculation [Dreaden et al., 2012]. Flocculation is the main limitation that poses a challenge for the synthesis of a stable GNPs colloidal solution. So, to avoid this

limitation and to synthesize a stable GNPs colloidal solution, polymers are employed in the formulation. With the advancement in nanotechnology, polymeric gold nanoparticles (P-GNPs) are extensively employed as drug nanocarriers for the targeted drug delivery. Direct, grafting to and grafting from methods are mainly followed in the synthesis of P-GNPs [Li et al., 2009]. Polymers generally used in the synthesis of P-GNPs are cellulose derivatives, chitosan, dextran, gelatin, guar gum, heparin, hyaluronic acid, maltose, polycaprolactone, polydiallyl dimethyl ammonium, polyethylene glycol, polyethylene imine, polystyrene sulfonate, polyvinyl caprolactam, polyvinyl pyrrolidone, pullulan, and xanthan gum are illustrated in Table 2.2. Graphical illustration of the distinction between GNPs and polymer stabilized GNPs are shown in Figure 2.2.

Table 2.2 Polymers used in the synthesis of polymeric gold nanoparticles [Adena et al., 2018]

Polymer	Nature
Cellulose derivatives	β linked D-glucose units
Chitosan	Linear polysaccharide, hydrophilic, cationic
Dextran	Branched polysaccharide, hydrophilic, cationic
Gelatin	From porcine skin, thermostable
Guar gum	Galactomannan, cationic
Heparin	Sulfated glycosaminoglycan, UV sensitive, anionic
Hyaluronic acid	Nonsulfated glycosaminoglycan, water soluble
Maltose	Disaccharide
Polycaprolactone	Film formation by thermal pressing
Polyethylene glycol	Biocompatible, non-toxic
Polystyrene sulfonate	Ion exchange resin, anionic
Polyvinyl caprolactam	Thermoresponsive
Polyvinyl pyrrolidone	pH- responsive
Pullulan	Amphiphilic, maltotriose units, hydrophilic, cationic
Xanthan gum	Repeated pentasaccharide, hydrophilic, anionic

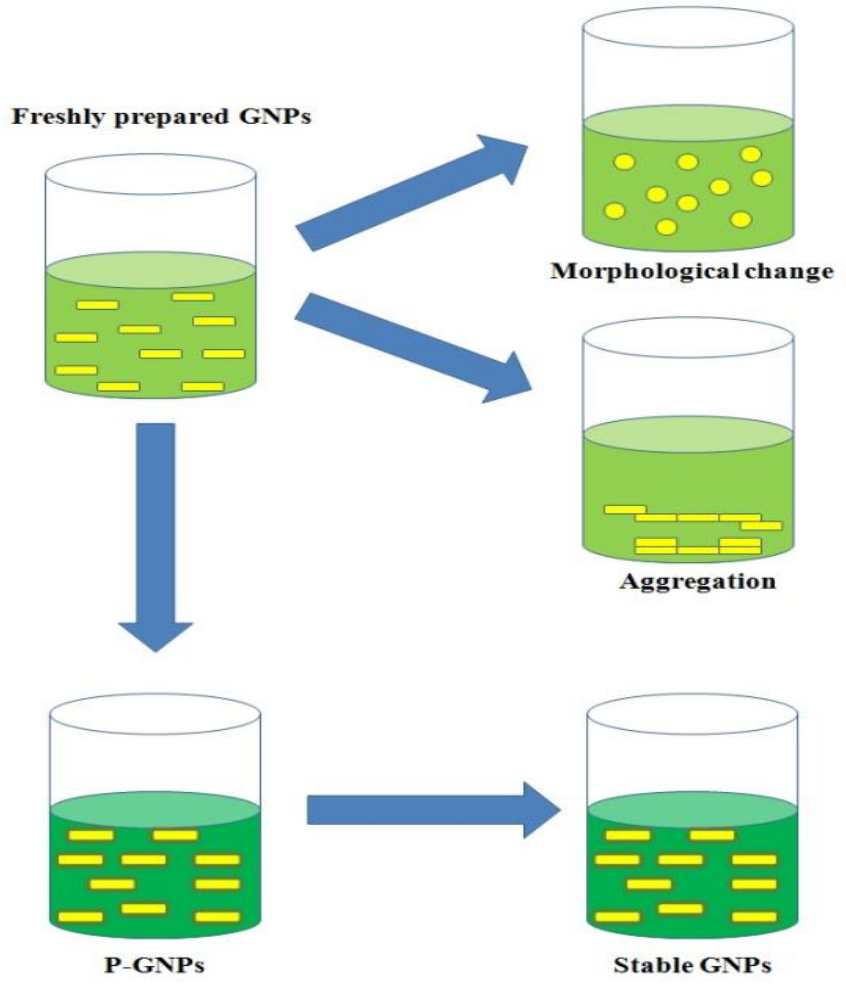


Figure 2.2 Graphical illustration of the distinction between GNPs and P- GNPs upon storage [Adena et al., 2018]

These polymers show a variety of advantages in cancer therapy viz., improving biocompatibility, imparting non-immunogenicity, improving the stability of GNPs, tuning of surface density, tuning of solubility and increasing the hydrophilicity of the outer surface [Danila et al., 2013]. In the direct synthesis method, tetrachloroauric acid is subjected to reduction in the presence of sulphur-terminated polymers to form P-GNPs in a single step [Scaravelli et al., 2013]. In grafting to method, functionalized polymers are attached to the surface of GNPs by polymers containing thiol or amine group. In grafting from method, GNP's surface is subjected to polymerization in the

presence of a chain transfer agent like thiocarbonyl compounds [Navarro et al., 2013, Takara et al., 2014].

Synthesis of controllable size P-GNPs is the prerequisite for its potential theranostic applications in the treatment of cancer, which could be possible by controlling the proportions of the polymers used in the formulation. Some polymers have both reducing and stabilizing properties,e.g., xanthan gum, polystyrene, and polyethylene imine, therefore, using these polymers as capping agents possibly will circumvent the use of reducing agents [Kirtane et al., 2013]. The advantages of P-GNPs to enhance the efficacy of the chemotherapeutic agent for the treatment of cancer are illustrated in Figure 2.3.

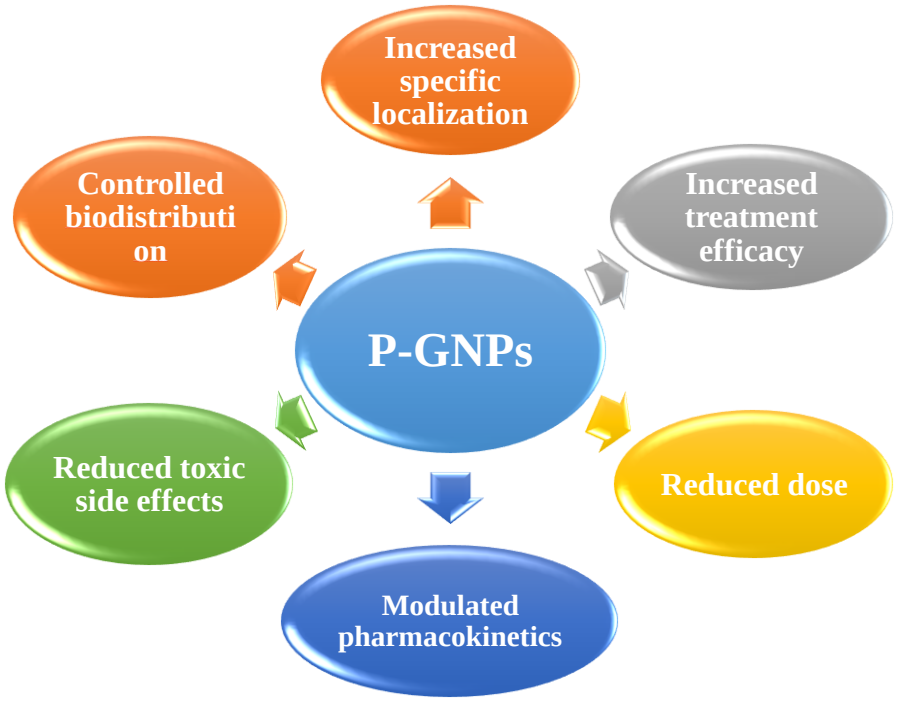


Figure 2.3 Advantages of P-GNPs to enhance the efficacy of chemotherapeutic agent for the treatment of cancer.

2.4.2 Physical methods

Gamma irradiation method is one of the best methods used for the GNPs synthesis with high purity and controlled size, where alginate solution which is a natural

polysaccharide is used as a stabilizing agent [Guo et al., 2014]. Bovine serum albumin protein is employed as a stabilizing agent for the synthesis of very small GNPs in gamma irradiation method [Deb et al., 2011]. By adopting heating or photochemical reduction method, GNPs are synthesized in which malate, citrate and tartrate ligands are used to reduce tetrachloroauric acid [Guo et al., 2012]. In the photochemical method, redox and polymerization reactions are used to synthesize gold-polyethylene glycol nanoparticles. In this method, glycine and tetrachloroauric acid solution were exposed to UV radiation wherein amino acid functionalized with glycine is used as photochemical initiator [Ganeshkumar et al., 2012]. In the microwave method, Cissus quadrangularis aqueous extract is used as a reducing agent [Pérez et al., 2011]. In the seed approach method, synthesis of stable GNPs is done by Co-60 irradiation using chitosan as a reducing agent. Synthesis of porous GNPs is done by using alloys of gold and silver where firstly micro emulsion of nanoparticles are prepared by using tetrachloroauric acid and silver nitrate and then reduced with sodium borohydride followed by de-alloying with nitric acid [Kojima et al., 2013].

2.4.3 Green methods

Chemical and physical synthesis strategies were proved to be harmful and may be injurious to humans as well as the environment due to the use of toxic chemical and elevated temperature in the synthesis process [Birla et al., 2009]. The green synthesis of GNPs is getting prevailing due to its non-toxicity and natural reduction at room temperature. This method emerges to be safe for clinical research as it is biocompatible, rapid, simple, suitable for large scale production, cost-efficient and eco-friendly [Rai et al., 2008]. There is a growing need to develop eco-friendly technologies in the field of nanotechnology to synthesize GNPs using micro-organisms and plants. A schematic

illustration of the mechanism of bioreduction and stabilization of GNPs is shown in Figure 2.4.

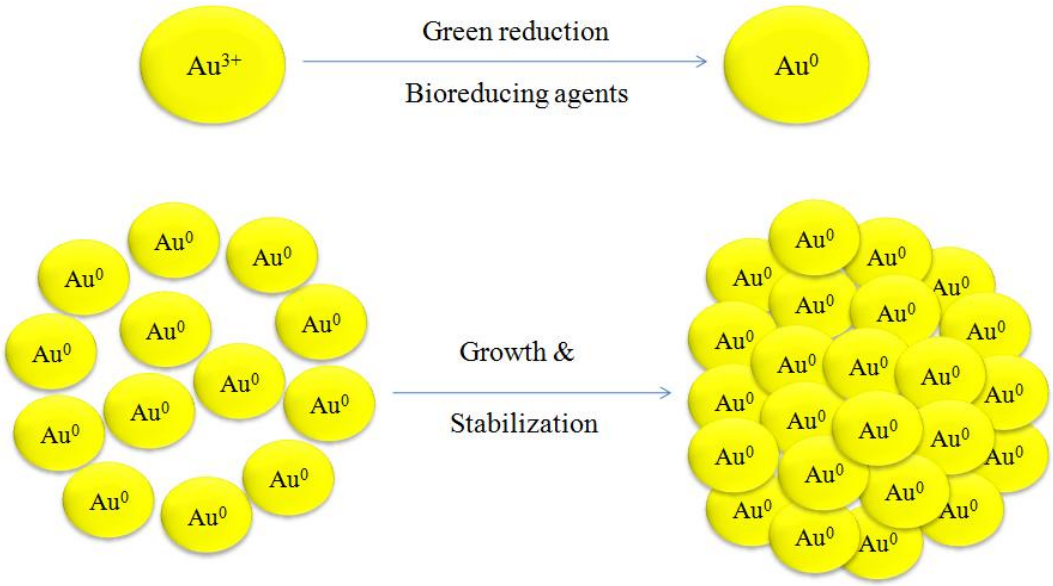


Figure 2.4 Schematic illustration of bioreduction and stabilization of gold into GNPs [Adena et al., 2018]

The biocompatibility is vital for GNPs for their theranostic applications. In GNPs green synthesis the addition of external stabilizing agents is not necessary as the biogenic components of microorganisms and plants act themselves as stabilizing as well as capping agents. An easy biosynthesis method for the preparation of GNPs is by using eggshell membrane. In this method eggshell membrane is immersed in the tetrachloroauric acid solution, to form stable GNPs. In another method, chitosan is used as a natural reducing and stabilizing agent to synthesize GNPs in aqueous sodium chloride solution [Krishnaswamy et al., 2015]. GNPs are also synthesized by using sunlight irradiation method in the reduction of gold salt is done by using solar energy. By using citrus fruits juice extracts which are obtained from Citrus reticulate, Citrus sinensis and Citrus limon [Tarnawski et al., 2011] tetrachloroauric acid is reduced to produce GNPs. Green synthesis of GNPs using different bioreducing agents are illustrated in Table 2.3.

Table 2.3 Green synthesis of GNPs using different bioreducing agents [Adena et al., 2018]

Bioreducing agent	Size	Shape
Azadirachta indica	11 nm	Spherical
Bacillus cereus	10-30 nm	Spherical
Butea monosperma	70-75 nm	Spherical
Micrococcus luteus	6-50 nm	Spherical
Streptomyces sp.	40 nm	Spherical
Brevibacterium casei	10-50 nm	Spherical
Mentha piperita	90 nm	Spherical
Bacillus stearothermophilus	5-30 nm	Triangular, spherical
Trichoderma viride	20-30 nm	Spherical
Zingiber officinale	5-15 nm	Spherical
Volvariella volvacea	20-150 nm	Triangular, spherical, hexagonal
Natural honey	15 nm	Spherical
Anacardium occidentale	36 nm	Hexagonal
Olea europaea	50-100 nm	Triangular
Hibiscus cannabinus	13 nm	Spherical
Mangifera indica	6-18 nm	Spherical
Coriandrum sativum	6-57 nm	Spherical
Nerium oleander	2-10 nm	Spherical
Solanum nigrum	50 nm	Spherical
Botrytis cinerea	1-100 nm	Triangular, spherical, hexagonal, decahedral and pyramidal

2.5 Controlling the size of the GNPs

All the unique properties of GNPs are dependent on their size and shape which can be managed by nucleation and growth processes during its synthesis. The size of the GNPs is usually governed by the aspects such as the nature and rate of addition of the reducing agent, temperature, nature of capping agents, and the ratio of reducing agent to capping agent. In the case of strong reducing agents like alkalides and hydrides, the rate of reduction is fast and leads to the formation of nucleation centers whose final size

depends on the nature of the capping agent. In the case of weak reducing agents such as sodium tartrate, trisodium citrate, and ascorbic acid the rate of reduction is slow and leads to the formation of large sized particles since the growth of the particle dominates the nucleation process. Seed growth method is used to synthesize particles of size more than 50 nm in a uniform manner having high poly-dispersity and varied shapes by eliminating nucleation [Brown et al., 2000].

2.6 Characterization of GNPs

GNPs are extensively characterized by various physico-chemical, morphological and solid state characterization techniques. Transmission electron microscopy (TEM) is useful for generating micrograph of the gold core of the GNPs and also provides the size distribution and dispersity of the sample. High resolution scanning electron microscopy (HR-SEM) and optical microscopy are useful for examining the core dimensions of the GNPs and atomic force microscopy (AFM) is useful for determining the surface morphology of the GNPs. Energy dispersive x-ray spectroscopy (EDXS) is useful to obtain the localized elemental composition of the GNPs in conjunction with the TEM. Selected area electron diffraction (SAED) is useful to explore the dispersity and crystallinity of the GNPs. UV-Visible spectroscopy is useful for analyzing the colloidal GNP dispersions having characteristic surface plasmon absorption. Fourier transform infrared (FTIR) spectroscopy and x-ray diffraction (XRD) are useful in detecting the order-disorder transitions in GNPs in the solid state [Adena et al., 2018].

Apart from these extensively used techniques, alternatively there are few less commonly used techniques for the characterization of GNPs. Scanning tunneling microscopy (STM) and small-angle x-ray scattering (SAXS) are used to determine the core dimensions of the GNPs. Differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), and thermo gravimetric analysis (TGA), are used to detect

the order-disorder transitions in GNPs in the solid state. To determine the interatomic distances and coordination numbers of the gold atom in the colloidal dispersion extended x-ray absorption fine structure (EXAFS) spectroscopy is used. To determine the chemisorptive properties of the ligands and the oxidation states of GNPs x-ray photoelectron spectroscopy (XPS) is used [Guo et al., 2016, Scott, 2015].

2.7 GNPs based theranostics

2.7.1 Labelling and visualizing

GNPs are mainly used for labelling applications, where they provide contrast for the examination and visualization by directing and enriching them at the region of interest. GNPs can be visualized with a wide variety of techniques, which makes them exceptionally appealing contrast agent. GNPs substantially tend to absorb and scatter visible light. A surface plasmon is observed in which the free electrons present in the GNPs get excited by the light energy and upon light absorption, the electrons show collective oscillation in the GNPs [Clavero et al., 2014]. Transfer of energy to the gold lattice results in relaxation of the excited electrons and the light absorption leads to heating of GNPs.

The visualization of the particles in numerous ways is possible mainly due to the interaction of GNPs with the light. GNPs of diameter more than 25 nm are directly imaged by using differential interference contrast (DIC) or optical microscopy in phase contrast mode [Aioub et al., 2014]. GNPs can be used for labelling with different colors, which depends on their particle's size and shape [Tang et al., 2015]. In the case of small GNPs, the cross section for absorption decreases slowly whereas for scattering it decreases rapidly. Absorption of the light results in heating of the GNPs which subsequently leads to heating of the GNPs environment. This is observed by photothermal imaging and photoacoustic imaging. The density fluctuations of the GNPs

are recorded by DIC microscopy in the former and expansion of the liquid environment due to the absorption of heat is recorded in the latter [Leduc et al., 2013].

Due to light absorption, a local heat pulse is generated, resulting in the expansion of the GNP's environment which ultimately leads to the generation of a sound wave that can be detected with the help of a microphone. Small GNPs tends to emit fluorescence upon photoexcitation which can be visualized by using fluorescence microscopy [Yahia-Ammar et al., 2016]. Apart from the visible light interaction, the electron waves and X-rays interaction is used as a valuable tool for the visualization of GNPs. Due to the high atomic weight of GNPs, they give high contrast in TEM. GNPs are used as X-ray contrast agents as they can efficiently scatter X-rays. With the help of neutron activation, GNPs can be radioactively labelled and can be detected by gamma radiation [Comenge et al., 2016].

2.7.1.1 Immunostaining

From a long back GNPs have been used in biology for immunostaining wherein by using antibodies the specific targeted components of outside and inside of the cells can be labelled. They can't be visualized without labelling as they lack contrast. GNPs conjugated with antibodies that are specific to the sample components are added to the predetermined and permeabilized cells. The GNPs provide brilliant contrast for TEM imaging and with optical microscopy larger structures can also be imaged [Marasini et al., 2013]. Even without fixing and permeabilizing the cells immunostaining is possible, but only labelling of the structures on the cells surface can be done. In this case, PAI (photoacoustic imaging) provides an additional characteristic in contrasting besides the above mentioned imaging techniques. When GNPs approach together, the plasmon resonance frequency tends to shift to higher wavelengths. When compared to freely scattered single GNPs, small aggregates of GNPs absorb the light at wavelengths above

the plasmon resonance. GNPs generate a photoacoustic signal when GNPs conjugated with antibodies binds to the receptors on the cell surface, unlike the GNPs which arbitrarily distributed on the surface of the cells [Mallidi et al., 2015].

2.7.1.2 X-rays contrast agents

The concept used in immunostaining for visualizing the components of cells is also used for providing *in vivo* contrast to the organs in animals and human beings. When GNPs conjugated with antibodies or ligands are administered into the circulation system, they will bind to the specific organ through ligand-receptor interaction. They eventually provide contrast and resolve the structure of the desired organ through imaging. The main problem associated with GNPs as contrast agents is that only a small amount of GNPs gets the opportunity to bind to the specific organ while the significant amount of GNPs is eliminated through the eliminating organs of the body because of their short circulation time in the body.

By using CT, GNPs are imaged with a significant signal to voice ratio [Cole et al., 2015] so that the exposure time can be reduced leading to a reduction in the radiation harm to the surrounding healthy tissues (Table 2.4). The organs of the body are imaged for the treatment by the penetration of X-rays through the skin. Moreover, X-ray computed tomography setups are readily available in many hospitals and diagnostic centres. GNPs causes less damage when compared to the quantum dots that are used as fluorescent semiconductor detecting and imaging agents [Hoshino et al., 2014].

Table 2.4 Summary of GNPs imaging applications [Adena et al., 2018]

Imaging	Property Required	Shape suitable
X-ray	X-ray absorption, no morphology/ shape relationship	Spherical
Optical	Strong light scattering	Spherical
Fluorescence	Strong inherent fluorescence	Spherical, rod, shell
Photoacoustic	Strong absorption in the NIR window	Spherical, rod, cage
SERS	Strong electromagnetic field	Spherical, star

NIR- Near Infra Red; SERS - Surface Enhanced Raman Scattering.

2.7.2 Targeting and delivery

GNPs are used as drug nanocarriers for the delivery of therapeutic agents into the tumor cells from quite a long time. Therapeutic agents are adsorbed on the GNPs surface, and the entire conjugate is introduced into the tumor cells by means gene guns or particle ingestion. Eventually after its administration, the therapeutic agents will detach from the GNPs inside the tumor cells. DNA is adsorbed on the GNP's surface, and these nanobullets are introduced into the tumor cells for the ballistic influx of DNA [Ibraheem et al., 2014].GNPs ingestion into the tumor cells is either nonspecific or specific through receptor-ligand interaction and the main objective involved in this is to transfer and deliver the therapeutic agents adsorbed on the GNP's surface into the cells [Bazak et al., 2015]. To deliver the therapeutic agents from the GNPs into the cytosol, GNP's surface is encapsulated with membrane disruptive peptides [Ding et al., 2014].

The delivery of therapeutic agents into tumor cells through particle ingestion is mainly useful in gene therapy and drug targeting. In gene therapy, for the expression of corresponding proteins, DNA adsorbed on the GNP's surface is introduced into the tumor cells [Vial et al., 2016]. In drug targeting, for the delivery of anticancer drug specifically to the target tumor cells or its microenvironment, GNPs are conjugated with specific ligands. To facilitate these applications, there is no need to exploit any of the

unique properties of GNPs and their characteristics like inertness, stable, small and relatively easy to conjugate with ligands make them be used as a biocompatible and reliable means for targeted drug delivery [Li et al., 2015, Rana et al., 2012].

2.7.2.1 Photoablation therapy (PAT)

PAT is mainly grouped into photodynamic therapy (PDT) and photothermal therapy (PTT) [Kim et al., 2013]. In order to accomplish the penetration in the blood and the tissue, NIR light is usually used. In PTT the absorbed light is converted into local heat i.e., hyperthermia which shows a significant cytotoxic effect on the tumor cells and its microenvironment [Wang et al., 2014]. In PDT the photon energy is converted to generate ROS (reactive oxygen species) by the stimulation of photosensitizers to kill tumor cells and its microenvironment. The absorption of light by the GNPs results in the excitation of the free electrons and this excitation at the SPR eventually leads to the cumulative oscillation of the energized free electrons. The GNP's crystal lattice and the electrons interaction lead to the relaxation of the electrons and the heat energy is exchanged to the lattice and subsequently scattered to the surrounding environment [Hogan et al., 2014]. Apart from its various imaging methods, heating of GNPs in a controlled manner leads to manipulation of the surrounding tissues in various ways [Jiang et al., 2013].

Intracellular delivery and the molecular uptake which mainly depends on phagocytosis and endocytosis can be potentiated by inducing hyperthermia at the tumor site [Gormley et al., 2013]. Due to extreme sensitivity, even a small increase in the temperature by a few degrees leads to the cells death. Temperature above 38^0 C results in fever and above 42^0 C results in cell damage in humans and by making use of hyperthermia in a controlled way cancer can be treated. This is possible by coordinating the GNPs whose surface is conjugated with ligands that are specific to the receptors that

are over-expressed on tumor cells. When the locally enriched GNPs in the tumor cells are heated with the help of external stimuli, the tumor cells which are in the vicinity of the GNPs can be killed selectively [Choi et al., 2012]. In this way, we can enrich malignant tissues with GNPs and illuminate the tissue. Tumor cells can be destroyed locally by GNPs mediated heat [Lee et al., 2014] without exposing the whole organism to higher temperatures.

PDT is a light sensitizer stimulation therapy which comprises of a particular wavelength of drug activating light, a photosensitizer drug and oxygen [Gupta et al., 2013]. The stimulation of photosensitizer leads to energy transfer and eventually yields highly reactive oxygen species which imparts apoptosis and necrosis by inducing microvasculature damage [Chinen et al., 2015]. Unlike radiotherapy, PDT does not have any harmful impact on cells and tissues that are devoid of photosensitizer drug as the light used for the stimulation is nonionizing. So it can be used safely without causing any harm to the neighbouring healthy tissue after taking a multi-dosage regimen [Seo et al., 2014, Master et al., 2013]. By means of fluorescence resonance energy transfer mechanism, the self-quenching capacity of GNPs plays a major role in image guided PDT in annihilating the tumor.

2.7.2.2 Sensing

Besides using GNPs in labelling, visualizing and targeted drug delivery, they are also used in active sensor applications. GNPs acts as sensors and explicitly register the existence of analyte molecules to give a readout which specifies the amount of analyte. GNP based sensors have a significant impact in diagnostics because of its small size.

2.7.2.2.1 Quenching

When the fluorophores are in close vicinity to the gold surface, their fluorescence can be quenched and this can be used for competitive displacement sensor

strategy [Liu et al., 2013]. For quantitative identification of an individual analyte molecule, ligands are conjugated with the GNPs that distinctively bind to the analyte molecule. By saturating with the analyte molecules, the binding sites of the ligands get jammed and then these analyte molecules are customized with fluorophores. Their fluorescence can be quenched as they are firmly connected to the GNPs. The free analyte molecules dislocate the analyte molecules that are bound to the ligands in a constant dynamic equilibrium. When the concentration of the free analyte molecules is more, the fluorophore labelled molecules discharges from the GNPs surface and there will be no quenching. This strategy can be changed by using GNPs as quenchers for quantum dots which are substituted by analyte molecules [Zhu et al., 2016].

2.7.2.2.2 Surface plasmon resonance

GNPs are used in sensing mainly because of its most reliable intrinsic feature, plasmon resonance frequency [Monreal et al., 2015] that can be altered directly by coupling the molecules to the GNPs surface [Zheng et al., 2016]. When the GNPs are closely arranged, the plasmon resonance frequency drastically changes, and they form minute aggregates which can be used for colorimetric identification of the analyte molecules. This technique was developed by Mirkin and his associates and is considered as one of the most distinguished illustrations of the gold based sensor [Ma et al., 2014]. To find the DNA, this innovative assay was developed. Oligonucleotides correlated to the target sequence that was supposed to be identified was conjugated with GNPs. When the target sequence does not exist then the GNPs freely scatters and the colloidal solution seems red. When it exists, the GNPs bind to it by hybridization of DNA complimentary strands. The hybridization leads to the development of GNPs aggregates, resulting in the adjustment in the plasmon resonance and the solution shows up a blue/violet color. At the point when the sample is subjected to heat, a mismatch in

even a single sequence results in an altered melting temperature which causes a change in the color. The quantitative identification of DNA sequences is feasible with this idea, even at a very low concentration [Yin et al., 2014].

Apart from DNA assays, the same idea is also used for the quantitative identification of the metals or proteins [Ilkhani et al., 2015]. The enzymatic action can also be checked with the same idea [Hutter and Maysinger, 2013]. Other than the analytes detection, such color changes can furthermore be utilized to measure the lengths. Discrete areas of analytes can be linked to GNPs and the conformation changes in the analytes can be examined [Zijlstra et al., 2012].

2.7.2.2.3 Surface enhanced Raman scattering

With the help of Raman scattering, many analyte molecules are distinguished, owing to their characteristic spectra [Szymanski, 2012]. In Raman scattering, the scattering is inelastic and the scattered light show lower or higher energy than the incident light. When the scattered light has lower energy, the energy is stored in the analyte molecules and when it has higher energy, the energy is attained from the analyte molecules. This shift in the energy is characteristic for the entities in the analyte molecules and they possess a characteristic Raman spectrum which is used for their identification and detection. The scattering efficiency mainly depends on the incident light's wavelength. Characteristically Raman signals are relatively weak and for that reason, an adequate concentration of the analyte molecules is required to provide signals that are relatively enough. In surface enhanced Raman spectroscopy, the Raman scattering can be significantly enhanced by guiding the analyte molecules close to the surface of GNP's having a high curvature [Vendrell et al., 2013, Yamamoto et al., 2014]. Because of the GNP's plasmon resonance, the electric field near the GNPs will be stronger than the field strength of the incident light.

Binding of the ligands to the GNP's surface significantly enhances the Raman signal of the analyte molecules and helps in its identification [Conde et al., 2014, Patra et al., 2014]. Recent advancements comprise of GNPs customized with Raman active analyte molecules for the identification of proteins [Zhan et al., 2016] or DNA [Xu et al., 2015] and two photon excitation [Xiao et al., 2014]. Various shapes of GNPs and their respective applications in the field of medicine are illustrated in Table 2.5.

Table 2.5 Shapes of various GNPs and their applications in the field of medicine [Adena et al., 2018].

Shape	Applications
Branched particle	Substrates for surface enhanced raman spectroscopy which could be utilized for imaging at the single molecule level.
Hollow particle	Photothermal cancer therapy, drug delivery, optical imaging, and catalysis.
Faceted particle	Catalytic activity, effective and reproducible substrates for SERS.
Nanocage	Contrast agent for optical coherence tomography (138) and endomicroscopy imaging.
Nanocube	Catalysis and field enhancement applications.
Nanorod	Selective biomarkers in diagnostics and selective targeting in photothermal therapeutics.
Triangular particle	Optical sensor application and filed enhancement.

2.8 Nanotechnology commercial impact

The research and development in the field of nanotechnology is continuously growing its importance both in terms of public and private funding. The global demand for the medicinal products developed by nanotechnology grew more than 15 % from 2009 to 2014. National Nanotechnology Initiative (NNI) is a US federal government program established in 2001 in collaboration with federal agencies and cabinet level departments to promote nanotechnology research, development and commercialization.

Since its inception, it received investments of more than $20 billion in support of transferring world class user facilities for characterization, modeling, and fabrication of nanotechnology based products, and cutting edge research from lab to market. For the fiscal year 2015, US federal government provided a funding of $1.5 billion to the NNI. Apart from US, the developed nations like Japan and the European Union are also investing extensive resources in nanotechnology. With the present pace of research and development nanotechnology innovations appears to be a front runner in the medical field and the returns are likely to exceed the investment [Pedrosa et al., 2015].

Nanocarrier based drug delivery is one of the advanced areas in the field of medicine, providing a broad range of novel formulations that are at the phases of pre-clinical or clinical trials. Nanomedicines are facing quite a few hurdles to enter the clinics like the conventional therapeutics due to lack of protocols for the characterization of nanomedicines in terms of pharmacokinetics and toxicity. On the other hand, the Nanotechnology Characterization Laboratory, in collaboration with the National Institute of Standards and Technology and U.S. Food and Drug Administration, has developed scientific tests that would help to determine the safety, efficacy, and reproducibility of nanomedicines to facilitate regulatory review. With this initiative, the European Union has furthermore set numerous goals in terms of patenting, business creation, and regulation for the nanotechnology products with international collaboration being a key benefit to improving research and development. In the Horizon 2020, the biggest European Union Research and Innovation Program, nanotechnology is included as one of the sections which show the commitment towards nanotechnology and its significance in increasing Europe's competitiveness.

At present nanotheranostics are still at an early stage of development and, thus, their impact on health expenditure and cost effectiveness is still complex to predict. Due

to low yield together with high expenses in research and development, and high competition among market players most of the companies prefer to invest in specific end use applications. Flourishing examples include Cytimmune (Aurimune®) and Nanospectra (Aurolase®) whose flagship products are now facing clinical trials. Even though the market for GNPs is still budding, their financial and clinical benefits are uncompromising. Best efforts should be made in the future to develop the connection between academia, research and development companies, pharmaceutical industry and the regulatory agencies.

2.9 Drug and excipients

2.9.1 Dasatinib

IUPAC name: N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-yl]amino]-1,3-thiazole-5-carboxamide.

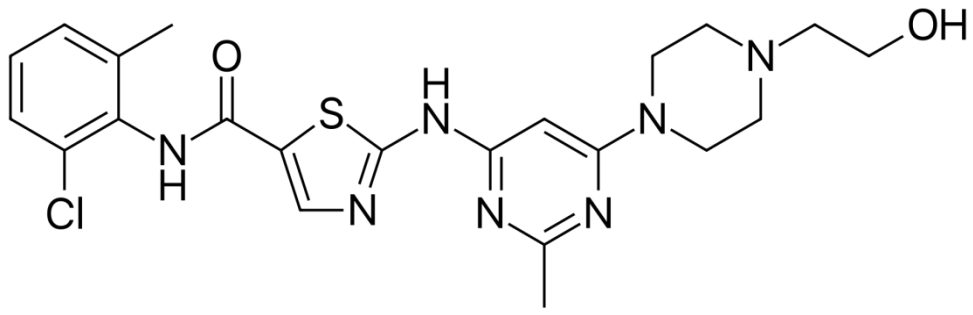
Molecular formula: $C_{22}H_{26}ClN_7O_2S$

Molecular weight: 488.007 g/mol

Polarity (log P): 1.8

Melting point: 280-286 °C

Chemical structure:



Description: DSB is a white to off-white powder.

Solubility: DSB is insoluble in water, slightly soluble in methanol and ethanol and freely soluble in DMSO.

Storage conditions: DSB should be stored at or below -20 ºC.

Mechanism of action:

DSB inhibits the BCR-ABL and Src kinases at the nanomolar concentrations. It binds to multiple conformations of the ABL kinase which was predicted based on modeling studies. Based on the *in vitro* studies, it was found that DSB was active in variants of imatinib sensitive and resistant leukemic cell lines. It inhibits the growth of BCR-ABL over expressing cell lines of CML and acute lymphoblastic leukaemia (ALL). DSB was able to overcome the resistance to imatinib which results due to the mutations of BCR-ABL kinase domain, activation of alternate signaling pathways involving the Src kinase, and MDR gene over-expression.

Therapeutic indications:

DSB is used to treat adults with CML (chronic, accelerated, or blast crisis phases) having intolerance or resistance to prior therapy. It is also used to treat adults with Ph+ (Philadelphia chromosome positive) ALL with intolerance or resistance to prior therapy.

Pharmacokinetics:

The pharmacokinetics of DSB showed dose proportional increase in the AUC (area under the plasma drug concentration time curve) and linear elimination characteristics over the daily dose range of 15 mg to 240 mg which was equal to 0.15 times the lowest and 1.7 times the highest of the approved recommended doses respectively. At 100 mg/day, the Cmax (maximum plasma concentration) at steady state was 82 ng/ml and AUC is 397 ng.hr/ml. The clearance of DSB was found to be time invariant.

Half life: 3-5 hours

Bioavailability: 14% (Oral)

Apparent volume of distribution: 2505 L

Protein binding: 96%

Metabolism: Hepatic

Elimination: Fecal and renal

Absorption: Following oral administration, Cmax of DSB was observed between 30 minutes and 6 hours. A single dose of 100 mg followed by a high fat meal (total calorie content - 985 kcal) increased the mean AUC of DSB by 14%.

Distribution: The V_D (apparent volume of distribution) was found to be 2505 L. Binding of DSB to active metabolite and human plasma proteins *in vitro* was approximately 93% and 96% respectively, without any concentration dependence over the range of 100-500 ng/ml. The mean plasma terminal half life and apparent oral clearance of DSB was found to be 3-5 hours and 363.8 L/hr respectively.

Metabolism: DSB is extensively metabolized in humans, primarily by cytochrome P450 enzyme 3A4 (CYP3A4). Apart from FMO-3 (Flavin containing mono-oxygenase 3) and UGT (uridine diphosphate glucuronosyltransferase) enzymes, CYP3A4 was the prime enzyme responsible for the formation of the active DSB metabolites. The active metabolites of DSB are equipotent to DSB and are unlikely to play a major role in the observed pharmacology and represents approximately 5% of the AUC of DSB. It also has numerous other inactive oxidative metabolites.

Excretion: Feces is the primary route of elimination of DSB. Following a single oral radio labelled dose of DSB, within 10 days 85% of the administered radioactivity was recovered in the faeces and 4% in the urine. Unchanged DSB accounted for 19% of the

administered dose in the feces and 0.1% in the urine with the remaining of the administered dose being metabolites.

Toxicological profile:

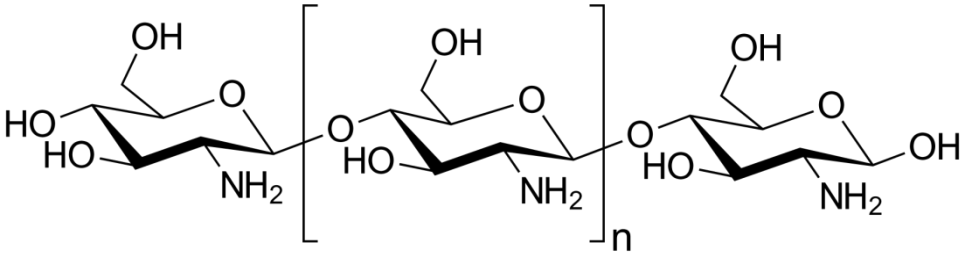
In a 2 year carcinogenicity study, DSB doses of 0.3, 1, and 3 mg/kg/day were administered to rats orally. The highest dose resulted in AUC level around 60% of the human exposure is at 100 mg/day. DSB induced a significant increase in the collective occurrence of prostate adenoma in low dose males and squamous cell carcinomas and papillomas in high dose females. When tested *in vitro* in Chinese hamster ovary cells DSB was found to be clastogenic. When tested in an *in vivo* rat micronucleus and in an *in vitro* bacterial cell, DSB was found to be non genotoxic and non mutagenic respectively. When tested in male and female rats at 100 mg daily (AUC similar to the human exposure), it was found that DSB did not affect fertility or mating of rats. Uterine mineralization and inflammation was observed when DSB was administered in monkeys and ovarian hypertrophy and cystic ovaries was observed when administered in rodents.

2.9.2 Chitosan

Chitosan is one of the most abundant natural polymer obtained by deacetylation of chitin which is the structural element in the exoskeleton of crustaceans (such as shrimp and crabs) and cell walls of many other species such as insects and fungi. It is broadly used in the delivery of proteins, peptides and polynucleotides due to the fact that it maintains the activity, structure and protect from the degradation. It is hydrophilic, biocompatible, biodegradable and prolongs the *in vivo* circulation time of the components [Tarane et al., 2009]. It features unique inherent physico-chemical properties which can be tailored to enable different pharmacological activities [Dobrovolskaia and McNeil, 2007]. Its use in the nanomedicine is getting prevailing

due to various advantages like cost-effective, eco-friendly, suitable for large scale production, sustained release property and tumor inhibiting property [Singh et al., 2016].

Chemical structure:



Chemical name: Poly-b-(1,4)-2-Amino-2-deoxy-D-glucose.

Molecular weight: 10,000 to 10,00,000 daltons.

Description: Chitosan is a white or creamy-white odourless powder or flakes.

pH: 4.0 - 6.0

Density: 1.35 - 1.40 g/cm^3

Glass transition temperature: 203 ^{0}C

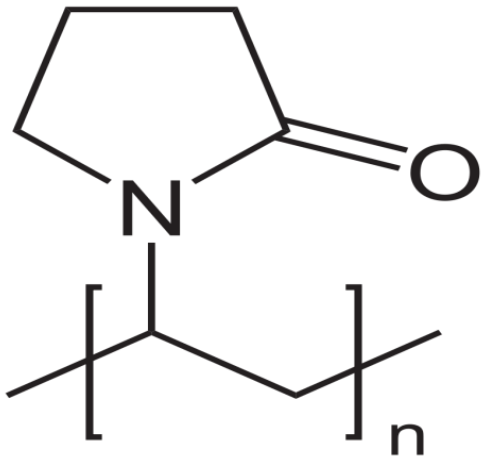
Solubility: Chitosan is readily soluble in most organic acids (dilute and concentrated solutions), sparingly soluble in water, and practically insoluble in ethanol and other organic solvents.

Storage conditions: Chitosan should be stored in a securely closed container in a cool and dry place.

Incompatibility: Chitosan is highly incompatible with strong oxidizing agents.

Safety: Chitosan is nontoxic, non-irritant, biocompatible and biodegradable. It is widely used as an excipient in pharmaceutical formulations and cosmetics.

2.9.3 Poly vinyl pyrrolidone (PVP)



Chemical Names: N-Vinyl-2-Pyrrolidone, N-Vinylpyrrolidone, 1-Vinyl-2-pyrrolidone, 1-vinylpyrrolidin-2-one.

Molecular weight: 111.144 g/mol.

Description: It is a white powder, compatible with a wide range of hydrophilic and hydrophobic resins.

pH: 3.0 - 7.0

Density: 1.23 to 1.29 g/cm^3

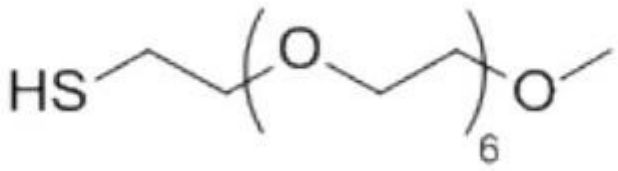
Melting point: 150 ^{0}C

Solubility: It is readily soluble in water and ethanol, and practically insoluble in ether.

Storage conditions: It should be stored in a securely closed container, in a cool and dry place separated from strong acids.

Safety: It has been verified to be of low concern based on experimental and modeled data and is widely used as an excipient in pharmaceutical formulations.

2.9.4 Poly ethylene glycol (PEG) thiol



Chemical name: Poly(ethylene glycol) monomethyl ether thiol, Methoxypolyethylene glycol thiol.

Molecular weight: 2000 daltons.

Description: It is a white or creamy-white odourless crystals or powder or chunks.

Melting point: 50-55 ^{0}C

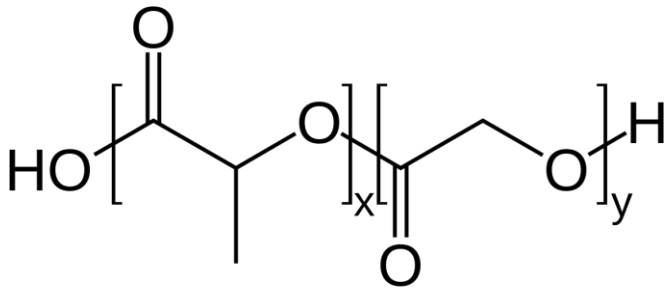
Solubility: It is soluble in water, and practically insoluble in ethanol and other organic solvents.

Storage conditions: It should be stored in a securely closed container in a cool and dry place.

Incompatibility: It is highly incompatible with strong oxidizing agents.

Safety: It is nontoxic, non-irritant, biocompatible and biodegradable. It is widely used as an excipient in pharmaceutical formulations and cosmetics.

2.9.5 Poly(lactic-co-glycolic acid) (PLGA)



Chemical name: 3, 6-dimethyl-1,4-Dioxane-2,5-dione.

Molecular weight: 90000 daltons.

Description: It is a white or creamy-white odourless crystals or powder or chunks.

Density: 1.30 g/cm^3

Glass transition temperature: 50-55˚C

Solubility: It is practically insoluble in water and soluble in organic solvents.

Storage conditions: It is susceptible to hydrolysis in the presence of moisture. Hence, it should be packed under high purity dry nitrogen and stored in airtight containers, preferably refrigerated at less than 8˚C.

Safety: It is nontoxic, non-carcinogenic, non-teratogenic, biocompatible and biodegradable. It is widely used as an excipient in parenteral pharmaceutical formulations.