DRUG DELIVERY SYSTEM OF DICLOFENAC SODIUM AND ITS APPLICATIONS IN THE TREATMENT OF INFLAMMATION AND EDEMA

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Abstract

Nanoparticles with a drug basis have emerged and caught attention in recent past because of its use in drug synthesis. Nanoparticles are reported for their role in enhancing bio-availability, reducing side effects in case of toxic drugs and extending the drug release. Diclofenac sodium (D.sod) is used for the treatment of inflammatory diseases. Excessive use of D.sod leads to severe side effects such as renal insuffiencecy and mucosal bleeding. This study was conducted to synthesize the pegylated diclofenac sodium gold nanoparticles (PEG-DAuNps). In-vitro anti-inflammatory activity is carried out using human red blood cell membrane (HRBC) stabilization method and egg albumin induced paw edema method is conducted for in-vivo anti-inflammatory analysis. DAuNps nanoparticles were prepared using chemical reduction method. The characterization of PEG-DAuNps was done by Fourier Transform Infrared Spectroscopy (FTIR) and Ultraviolet-Visible Spectroscopy. The average size of prepared nanoparticles is 26nm.In-vitro release study of PEG-DAuNps showed sustained drug release. PEG-DAuNps showed faster reduction in paw thickness of mice. PEG-DAuNps showed higher protection towards HRBC membrane. PEG-DAuNps could be used as a potential therapeutic agent for the treatment of inflammatory diseases with reduced side effects.

Key Words: *Diclofenac sodium, Anti-inflammatory drug, Paw edema, Gold nanoparticles, Pegylation.*

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Abbreviations

D.sod	Diclofenac sodium
PEG	Polyethylene glycol
DAuNps	Diclofenac sodium gold nanoparticles
FTIR	Fourier Transform Infrared
Nps	Nanoparticles
SEM	Scanning Electron Microscopy
AuNps	Gold Nanoparticles
NSAIDS	Non-sterodial anti-inflammatory drugs
HRBC	Human red blood cell membrane
OD	Optical density
HAuCl4	Chloroauric acid
NaBH4	Sodium borohydride
HT	Histamine

CHAPTER 1: INTRODUCTION

1.1 Inflammation

Inflammation is the reaction of living cells to different types of trauma. It consists of well regulated series that involves cellular and fluidic changes. The cardinal features of inflammation are rubor (redness), tumour (swelling), calor (heat), dolor (pain) and functio leasa (loss of function).These four cardinal features were introduced (Celsus, Targa, & Bianconi, 1806). It has both useful and harmful effects systemically and locally. The acute and chronic inflammations have systemic effects on the body which includes fever, leukocytosis and endotoxemia. Inflammatory reaction is almost visible in all living organisms but some higher level of living organisms uses blood to deliver components such as fluid and cells into the extracellular space.

Inflammation is a very complex process. Inflammatory mediators have different functions having multiple effects on different types of tissues. There are different stages of inflammation such as peracute, acute, subacute and chronic. In the early stage of acute inflammation the infected tissue have swelling and redness because of edema and increased flow of blood vessels. Microbial infections such as bacteria, fungi, protozoa, viruses and various parasites, hypersensitivity reactions, corrosive chemicals, tissue necrosis are the main cause of acute and chronic inflammation.

Persistent cytokine release, swelling, inappropriate inflammatory response and normal tissues destruction are the harmful effects of inflammation. Inflammation is triggered by injurious stimulus and if it doesn't get repaired in the specific period of time (acute), it develops into chronic inflammation. Factors such as angiogenesis, mononuclear cell infiltrate and fibrosis which cause chronic inflammation. Blood vascular system is the main transport system to deliver inflammatory mediators into the extravascular space.

Inflammation is a main factor that aggravates many diseases such as rheumatoid arthritis, osteoarthritis, asthma, kidney and cardiovascular diseases. They are treated with non-steroidal and steroidal anti-inflammatory drugs. They exert their anti-inflammatory effects by preventing

the pathway of arachidonic acid with the help of lipoxygenase and cyclooxygenase enzymes (Insel, 1996).

1.2 Non-Sterodial Anti-Inflammatory Drugs (NSAIDS)

A group of drugs exert anti-inflammatory, analgesic and anti-pyretic effects. They inhibited the production of thromboxanes and prostaglandins by suppressing the cyclooxygenase enzymes such as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). NSAIDS exert anti-inflammatory, anti-pyretic and analgesic effects by inhibiting the COX-2 activity and by suppressing the COX-1 activity it leads to peptic ulceration, mucosal injury and bleeding. In ancient times NSAIDS were given to treat joint inflammatory diseases such as rheumatoid arthritis, osteoarthritis, sprains, bursitis by inhibiting non-selectively the COX activity. During the process of inflammation the protein activity and mRNA levels of COX-1 do not alter but a remarkable increase of COX-2 increases the synthesis of prostaglandins.

1.3 Diclofenac Sodium

Diclofenac sodium (D.sod), phenylacetic acid derivative, is a non-sterodial anti-inflammatory drug (NSAID) that exerts analgesic and anti-inflammatory activity and is being clinically used in the treatment of rheumatoid arthritis, osteoarthritis, and mild to moderate pain. D.sod blocks the conversion of arachidonic acid into prostaglandins by suppressing the cycloxygenase enzymes such as COX-1 and COX-2.It is a competitive inhibitor of proinflammatory prostaglandin E2 (PGE2) which induces inflammation and platelet aggregation (Ku et al., 1985).COX-1 and COX-2 is responsible for the protection of gastric mucosa. By suppressing the COX-1 and COX-2 activity it induces peptic ulceration and mucosal bleeding(Wallace, McKnight, Reuter, & Vergnolle, 2000). There are common side effects related with the excessive use of diclofenac such as renal failure, hepatotoxicity, skin rashes and gastrointestinal lesion formation (Sena, Chaudhry, Collins, & Poppi, 2004).Due to its shorter biological half-life and high protein

interaction, high doses of D.sod are required for the efficient therapeutic activity (Carson, Notis, & Orris, 1990).

1.4 Nanotechnology

Nanotechnology provides access to medical related research such as developing nanomedicine, which is also a growing market. Variety of drugs with nanoparticle formulations are reportedly increasing efficiency of drugs by intensifying the capacity of drug for better treatment, enhancing the solubility to improve effect and also controlling the drug release and preventing metabolism to provide long term effects. Research also implies of using nanomedicine which involves nanomaterials and devices for improvement in diagnosis and drug release mechanism (Szelenyi, 2012). To date, few drug delivery strategies for D.sod have been developed including D.sod loaded N trimethyl chitosan nanoparticles (Asasutjarit et al., 2015), poly(lactide-co-glycolide) (PLGA) based diclofenac loaded nanoparticles (Cooper & Harirforoosh, 2014) , D.sod composite microparticles (Jelvehgari, Barar, Valizadeh, Loveymi, & Ziapour, 2010). D.sod loaded iron ethyl celloluse (core/shell) nanoparticles (Arias, López-Viota, López-Viota, & Delgado, 2009). Solid lipid nanoparticles of D.sod have also been developed (Liu et al., 2010).

1.5 Gold Nanoparticles

Application of Gold nanoparticles can be seen in various fields, one major amongst other being biomedical field. The significance depends upon their properties such as control in drug delivery, role in treatment of cancer, biomedical imaging, diagnosis and etc. Due to their synchronization with human body, certainly low toxicity, adjustable stability and their flexibility to come in contact with a variety of substances. Gold nanoparticles have remarkable tendency of their use as drug delivery systems. Synthesis of gold nanoparticles can be done in multiple ways, most generalized being chemical and biological methods. First one offers improved control on size and shape of nanoparticles, thus secures an advantage over the other. Apart from this polyethene glycol-gold nanoparticles (PEG-AuNps) are prominent in nanomedicine because of their wide

range of applications, which involves imaging within the cell to drug delivery due to their nature of significant compatibility and extensive circulation time in blood.

It is reported that nanoparticles have been considered as promising carriers for anti-inflammatory drugs (Sahoo & Labhasetwar, 2003) with less cytotoxicity (Connor, Mwamuka, Gole, Murphy, & Wyatt, 2005). Gold or silver nanoparticles loaded heparin show no significant effect on systemic hemostasis due to its anti-inflammatory activity (Kemp et al., 2009).21nm gold nanoparticles inhibit the inflammatory effects by reducing the TNF- α and mRNA levels in mice (Chen et al., 2013). High doses of gold nanoparticles showed a significant reduction of proinflammatory cytokines TNF- α. The intra-articular administration of nanogold reduces the TNF-α and IL-1β levels, macrophage density, histological scores, microcapillary density and cell proliferation and migration in rat models of rheumatoid arthritis (Tsai et al., 2007).

1.6 Objectives

The aim of this study is to synthesize the pegylated D.sod gold nanoparticles (PEG-DAuNps) using chemical reduction method. Characterization of PEG-DAuNps was done using several techniques such as Ultraviolet-Visible Spectroscopy (UV-Vis spectroscopy), Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM).In-vitro anti-inflammatory activity was carried out using human red blood cell membrance (HRBC) stabilization method and egg albumin induced paw edema method was conducted for in-vivo anti-inflammatory analysis.

CHAPTER 2: LITERATURE REVIEW

2.1 Non-sterodial Anti-inflammatory drugs (NSAIDS)

NSAIDS, a group of anti-inflammatory drugs, has been generally used worldwide to treat several inflammatory diseases such as rheumatoid arthritis, osteoarthritis, artherosclerosis and cancers. They have anti-pyretic, anti-inflammatory and analgesic properties. NSAIDS are approved by Food and Drug Administration (FDA).The following non-sterodial anti-inflammatory drugs are diclofenac sodium, indomethacin, ibuprofen, naproxen, phenylbutazone, aspirin and piroxicam.The mechanism of action of NSAIDS such as aspirin through their prevention of proinflammatory prostaglandin synthesis was reported in 1971 (J. R. Vane, 1971).They reduce the synthesis of proinflammatory prostaglandins that causes pain, fever and inflammation. Weismann reported the side effects of aspirin when it inhibits the prostaglandin synthesis (Weissmann, 1993). High doses of NSAIDS cause major damage to kidney and stomach lining. The most common adverse effect of NSAIDS is gastrointestinal bleeding. There are two isoforms of cyclooxygenase enzymes such as COX-1 and COX-2 that synthesize the production of prostaglandins.COX-1 is triggered by physiological stimulus and it releases thromboxane A2, prostaglandin E2 (PGE2) and prostacyclin. Prostaglandin E2 (PGE2) is responsible for the protection of gastrointestinal tract by up regulating the mucosal blood flow, mucus and biocarbonate secretion (Wallace, 2008).Thromboxane A2 increases the platelet aggregation and induces vasoconstriction. Prostacyclin is sythesized by the endothelial cellwall and it has antithrombogenic properties (Moncada, Gryglewski, Bunting, & Vane, 1976). NSAIDS inhibited the COX-1 activity which leads to the gastrointestinal irritation, heart attack and strokes, Na and water retention, hypertension, and hemodynamic acute injury.COX-2 is triggered by the inflammatory stimulus and it releases the prostaglandins, inflammatory mediators and proteases which induces inflammation. NSAIDS prevents the COX-2 activity which inhibits the inflammation.

2.2 Diclofenac sodium

Diclofenac sodium, a phenylacetic acid derivative, a non-sterodial anti-inflammatory drug possesses anti-inflammatory, analgesic and anti-pyretic properties. It is commercially available

in the form of sodium salt. The molecular formula of diclofenac sodium is $C_{14}H_{10}Cl_2NNaO_2$.The molecular weight of diclofenac sodium is 318.129 g/mol. The enteric coated slow release tablet of diclofenac sodium was reported in Japan 1974 and it was used for the treatment of rheumatoid arthritis, osteoarthritis, mild to moderate pain and ankylosis spondylitis (Gan, 2010). The adverse effects have been associated with the high dosage of diclofenac sodium use such as hepatic, cardiovascular and kidney diseases. Chemical structure of diclofenac sodium is shown in Figure 1.

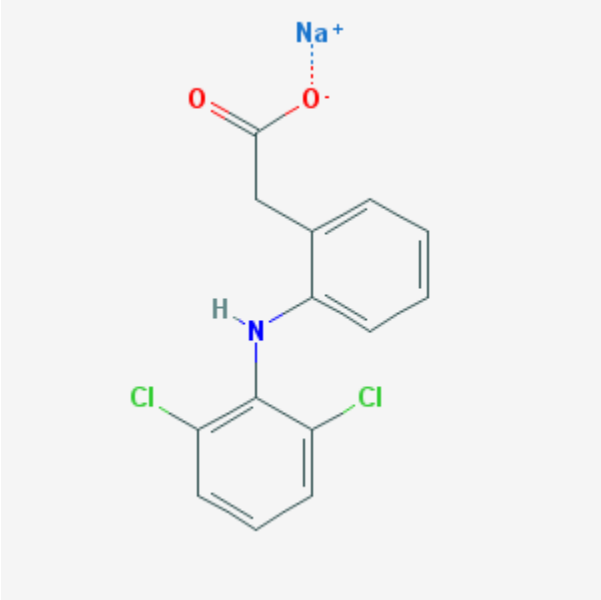

Figure 1: Chemical Structure of Diclofenac sodium

2.3 Anti-inflammatory effects of diclofenac sodium

The mechanism of action of diclofenac sodium is unidentified but there is some evidence which leads to the proven mechanism of action of non-sterodial anti-inflammatory drug. Vane proposed the mechanism of action of non-sterodial anti-inflammatory drug by blocking the cyclocxygenase to stop the synthesis of proinflammatory prostaglandins (J. R. Vane, 1971). This mechanism of action of NSAIDS is the most well known mechanism. Blocking of thromboxanes and prostaglandins production by non-sterodial anti-inflammatory drugs (NSAIDS) have been experimented in vivo and in vitro on animal models (J. Vane & Botting, 1996).Like Non-

sterodial anti-inflammatory drugs (NSAIDS), diclofenac sodium stops the conversion of arachidonic acid into prostaglandins and thromboxanes by inhibiting the cyclooxygenase enzymes such as COX-1 and COX-2 (Hecken et al., 2000),(Tegeder et al., 1999),(Wittenberg, Willburger, Kleemeyer, & Peskar, 1993). Diclofenac sodium is the most competitive inhibitor of proinflammatory prostaglandin such as prostaglandin E2 (PGE2) which causes inflammation. When cell membrane is exposed to the inflammatory stimulus phospholipids of the membrane, it releases arachidonic acid. Arachidonic acid is converted into proinflammatory prostaglandins and thromboxanes such as PGI2, PGE2, TXA2, PGD2, PGF2α, PGH2 which causes inflammation. Inhibition of conversion of arachidonic acid into proinflammatory prostaglandins by diclofenac sodium reduces the inflammation process. The anti-inflammatory pathway of diclofenac sodium is shown in Figure 2.The isoform of cycloxygenase enzyme such as COX-1 is more expressive in different types of tissues.COX-1 has more stability as compared to COX-2.It normally regulates renal blood flow, platelet function and protects the lining of gastrointestinal tract while COX-2 upregulates the production of inflammatory mediators such as leukotrienes, thromboxanes and prostaglandins which causes pain (Tegeder, Pfeilschifter, & Geisslinger, 2001). There are some other pathways which diclofenac sodium use to inhibit the inflammatory reaction. Diclofenac sodium inhibits the IL-6 expression and PGE2 synthesis in human T cells and chrondrocytes (Tsuboi, Tanaka, Nakao, Shichijo, & Itoh, 1995) (Henrotin et al., 2001). The IL-6 levels were decreased in plasma and synovial fluids of rheumatoid arthritis patients when it was treated with diclofenac sodium for 7 days (Sacerdote et al., 1995). Inactive Thromboxane B2 is a promoter for the synthesis of thromboxane A2 through the activation of COX-1 (Armstrong, 1996). Diclofenac sodium binds to the TP receptor (Thromboxane-prostanoid) to stop the synthesis of thromboxane A2 which is involved in inflammation (Selg et al., 2007). Diclofenac sodium is 3 to 1000 times potent as compared to other non-sterodial anti-inflammatory drugs (NSAIDS).The concentration of drug in plasma is related with the inhibition of prostaglandin E2 (PGE2) by diclofenac sodium (Giagoudakis & Markantonis, 2005). Diclofenac sodium is four fold more selective for COX-2 (IC 80:0.23 mM vs 1.0mM) (Warner et al., 1999).There is some evidence that the dual inhibition of cyclooxygenase enzymes such as COX-1 and COX-2 is associated with the gastrointestinal irritation.

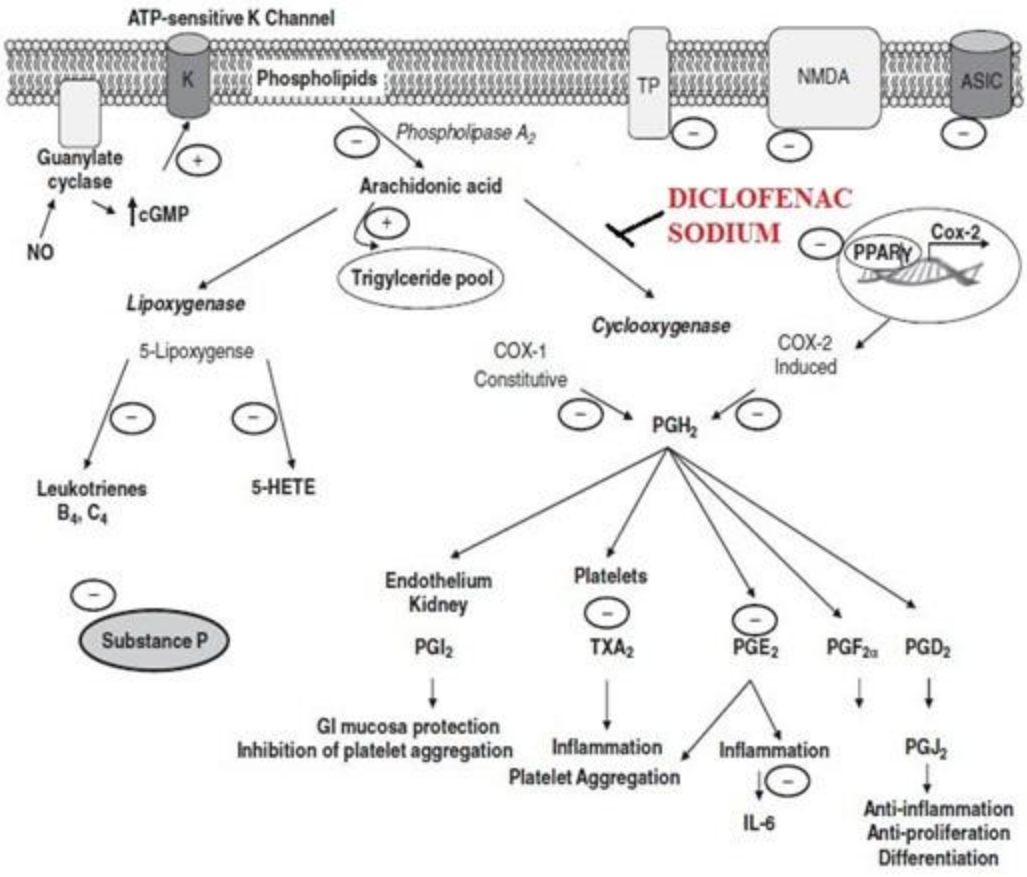

Figure 2: Schematic diagram of Anti-inflammatory pathway of diclofenac sodium (Gan, 2010)

2.4 Adverse effects of diclofenac sodium

Inhibition of COX-2 levels by diclofenac sodium results in main adverse effects such as renal failure and hepatotoxicity .By inhibiting the COX activity, the myeloperoxidase levels (MPO) is increased in intestine which causes inflammation and oxidative stress (Loria, Dato, Graziani, & Biasucci, 2008) (Hanifeh et al., 2015)

2.4.1 Gastrointestinal tract (GI) Complications

Treatment with diclofenac sodium causes low gastrointestinal tract (GI) complications such as peptic ulcers and mucosal bleeding. One third of high risk patients taking diclofenac sodium were associated with anemia, colitis, perforation and angiodysplasic bleeding (Chan et al., 2002; Lanas & Sopeña, 2009).

2.4.2 Cardiovascular risk

Recent studies have reported that there is a high chance of stroke and myocardial infarction when it is treated with diclofenac sodium. In one case study osteoarthritis patients receiving diclofenac sodium had an increased chance of thrombotic cardiovascular risk (Combe et al., 2009).

2.4.3 Renal failure

Low therapeutic dose of diclofenac sodium do not cause renal failure in healthy adults (Dilger et al., 2002). However, some results have shown that continuous treatment decreases the creatinine clearance rate which is a marker of renal failure (Whelton, Lefkowith, West, & Verburg, 2006).

2.4.4 Liver injury

Diclofenac sodium increases the transaminase level which is a marker of liver failure as compared to other non-sterodial anti-inflammatory drugs (NSAIDS) (Moore, 2007; Rostom, Goldkind, & Laine, 2005).In 17,289 osteoarthritis and rheumatoid paitents receiving diclofenac sodium for 18 months, there is an increased level of aminotransferase which is involved in hepatotoxixity (Laine et al., 2009).

2.4.5 Side effects of topical and oral formulations

There are some side effects which is associated with topical diclofenac sodium formulations use such as skin allergic reactions (itching, burning and rashes) (Moen, 2009; Rainsford, Kean, & Ehrlich, 2008; Zacher et al., 2008).Topical diclofenac sodium formulations have an increased incidence of gastrointestinal tract (GI) and cardiovascular risk whereas oral formulations of diclofenac sodium is associated with renal failure and hepatotoxicity.

2.5 Nanotechnology

Over the period of twenty years, nanomaterials (NM) have extensive applications in research field. Nanotechnology has expanded its boundaries for better treatment, immunogenicity, early diagnosis, better drug pharmacokinetics, nonspecific toxicity, controllable drug release and targeted drug delivery. There are different types of nanoparticles such as liposomes, solid lipid nanoparticles, nanocrystals, nanotubes and polymeric structures (Khanbabaie & Jahanshahi, 2012). Nanocrystals increase the solubility and bioavailability of poorly soluble medications by improving the pharmacokinetics and pharmacodynamics properties (Gao et al., 2013; Yadollahi, Vasilev, & Simovic, 2015). They can be designed to protect the encapsulated drug which is a solution for poorly soluble drugs (Kayser, Lemke, & Hernandez-Trejo, 2005). They allow specific drug delivery to target the mononuclear phagocyte system (MPS) and minimize the adverse effects (Brigger, Dubernet, & Couvreur, 2002). Drug delivery systems with narrow range of particle size have high bioavailability. They bind easily with cells and biomolecules because of their unique chemical and physical properties such as solubility, particle size, shape, surface structure, particle aggregation and agglomeration. They interact with nobel metal elements such as Ag, Au, Fe, Pt. Nonetheless, it is necessary to check the safety of this new technology on living systems before dispersing into the blood. Because of having large surface to volume ratio and small dimensions, nanomaterials cause irreversible injuries when it enters into lymphatic and circulatory systems through oxidative stress. Silver nanoparticles cause genotoxicity and oxidative stress in cell lines and animal tissues (Hornos Carneiro & Barbosa Jr, 2016).

2.5.1 Diclofenac sodium delivery approaches

Diclofenac sodium is the most prescribed non-sterodial anti-inflammatory drug. Due to its short half life as 1-2 hours when administered orally it absorbed quickly (Sweetman, 2009).High doses of oral diclofenac sodium is required to achieve more therapeutic response and it leads to the gastrointestinal complications. Therefore nanocarriers have been developed to increase the half life of diclofenac sodium. Diclofenac sodium loaded Magnetic nanoparticles have been prepared to obtain the prolonged drug release and 11% drug loading levels (Agotegaray & Lassalle, 2014).

Different types of controlled diclofenac sodium delivery approaches have been attempted to increase the absorption of drug (Kaur & Harikumar, 2012) (Table 1).

NSAIDS	**Delivery approaches**	**Importance**
DICLOFENAC SODIUM	Pellets	Enhance bioavailability
	Soft gels	Less Adverse effects
	Microspheres	Better targeting
	Nanocomposites	Prolonged drug release profile
	Microcapsules	Good efficacy
	Suppositaries	Less gastric complications
	Liposomes	Increase skin delivery
	Pharmacosomes	High drug loading

Table 1: Delivery approaches of diclofenac sodium

These drug delivery approaches could not minimize the gastrointestinal tract (GI) complications. Therefore, new safe targeted therapy is required to minimize the adverse effects. Nanocomposites are a better delivery approach due to its unique properties such as prolonged drug release profile, more extention time in blood, less toxicity and highly stable. To improve this approach, diclofenac sodium coated gold nanoparticles will be formulated to minimize the gastrointestinal and renal side effects.

2.6 Gold Nanoparticles

Gold nanoparticles have broad spectrum in biomedical applications. Eastern physicians used gold for medicinal purpose in the treatment of various diseases (Higby, 1982). The anti-inflammatory properties of gold salts was reported in the treatment of rheumatoid arthritis

(Cecil, 1940). Gold nanoparticles are found to be promising nanocarriers for the treatment of inflammatory diseases due its anti-inflammatory properties with less toxicity. Gold nanoparticles are highly stable, low toxic, good conductor and more biocompatible. They easily bind with therapeutic drugs due to large surface area. Gold nanoparticles can be prepared by two methods such as biological and chemical method. Chemical method is a simple and preferential method because it easily controls the shape and size of particles.

2.6.1 Anti-inflammatory properties of Gold nanoparticles

Initially, Gold salts have been used for the treatment of arthritis such as aurous sodium thiopropanolsulfonate, aurous sodium thiosulfate, aurous sodium thiomalate, aurous thioglucose,aurous potassium cyanide (Davis, Johnston, Miller, & Wong, 1983; Fumagalli et al., 2002; Graham, Champion, & Ziegler, 1994). The anti-inflammatory pathways of gold salts are not fully understood but they are related to the prevention of prostaglandins synthesis (Stone, Mather, & Gibson, 1975) and lysosomal enzymes release with the help of phagocytic cells (Dimartino & Walz, 1977; Westwick, Allsop, & Watts, 1974). Gold salts helps to prevent the formation of pannus by inhibiting the collagen synthesis and synovial cells proliferation which causes inflammation (Goldberg, Parrott, Kaplan, & Fuller, 1980). Another anti-inflammatory mechanism of action of gold salts involves the inhibition of reactive oxygen species synthesis such as hydroxyl radical, hydrogen peroxide (Miyachi, Yoshioka, Imamura, & Niwa, 1987) and superoxide anion (Davis, Johnston, Miller, & Wong, 1983).

The anti-inflammatory mechanisms of gold nanoparticles are shown in Table 2.

S.No.	**Anti-inflammatory mechanisms of AuNps**
1.	Decrease the HIF-1α expression, TNF-α and P13 K levels in blood
2.	Decrease the IFN-1γ, IL-4, IL-17 and TNF-α levels in blood and death of $CD4^+$ T cells
3.	Downregulate the IL-2, IL-6 and pERK levels
4.	Decrease the TNF-α, IL-1β and myeloperoxidase (MPO), redox state b reversible
5.	Decrease the TNF- α, IL- β1 levels, edema and macrophage accumulation in the infected area
6.	Increase the Catalase b levels

Table 2: Anti-inflammatory mechanism of AuNps

CHAPTER 3: MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich. Distilled and deionized water were used throughout this study.

Male and female BALB/c mice were purchased from National Institute of health (NIH) sciences.

3.1 Synthesis of diclofenac sodium gold (DAuNps) nanoparticles

D.sod gold nanoparticles (DAuNps) were prepared using chemical reduction method. Briefly, 1mM solutions of both D.sod and HAuCl4 were prepared in distilled water. The pH value of D.sod solution was adjusted to around 6.NaBH4 solution (40 mM) was dissolved in deionized water. A mixture of D.sod (5 ml) and HAuCl4 (1 ml) were heated and stirred in a waterbath at 50°C and 200 rpm for 20 min.0.05 ml of chilled NaBH4 was added to the mixture in a drop wise manner. The mixture was kept on shaking for 5 h at a rate of 200 rpm resulted in the formation of nanoparticles. Different ratios, temperature and pH were optimized for the synthesis of DAuNps (Table 3).The optimized DAuNps were dried using Freeze dryer (Eyela FDU-1000) for 24 h at 40 Pa.

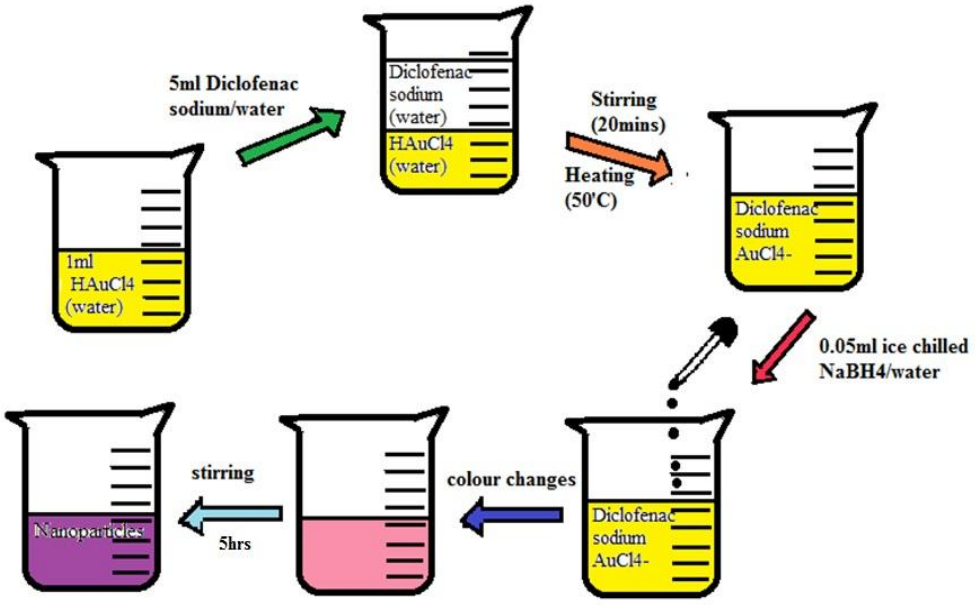

Figure 3: Synthesis of Diclofenac sodium coated gold nanoparticles by chemical reduction method

S.No.	PARAMETERS						
1.	D.sod Concentration (ml)	1	2.5	5	10	15	20
2.	HAuCl4 concentration (ml)	1	2.5	5	10	15	20
3.	NaBH4 concentration (ml)	0.05	0.1				
4.	Temperature	25ºC	37ºC	50ºC	75ºC	100ºC	
5.	pH	4	6	8	10		

Table 3: Variation of parameters for the optimization of nanoparticles

3.2 Synthesis of PEG coated DAuNps (PEG-DAuNps) nanoparticles

Freeze dried DAuNps (2.5 mg) were dissolved in distilled water under vigorous stirring for 30mins.The solution was centrifuged at 7000 g for 1 h and supernatant and the pellet were separated. The supernatant and the pellet were recorded using UV-Vis spectrophotometer. PEG-6000 (2.5%) solution was prepared in deionized water.PEG-6000 (2.5%) solution was then added drop by drop to the purified DAuNps suspension(dissolved in distilled water) under vigorous stirring for 30 min. The pellet of PEG-DAuNps was collected after centrifugation at 7000 g for 1 h.

3.3 Ultraviolet Visible Spectroscopy (UV-Vis)

UV-Vis spectra of D.sod, HAuCl4 and DAuNps were recorded by Uv-vis 2800 BMS Scientific Technical Corporation (PVT) Ltd. This shows the absorbance spectra of gold nanoparticles

3.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analyses were made after the preparation of test samples using compressed KBr discs. FTIR spectra of D.sod and DAuNps were individually measured between 3500 cm^{-1} and 500 cm^{1} using the FTIR spectrophotometer (Perkin Elmer spectrum 100 instrument).

3.5 Scanning Electron Microscopy

PEG-DAuNps were dissolved in distilled water prior to SEM imaging. Images were observed using Scanning Electron Microscopy.

3.6 Drug encapsulation efficiency

The amount of drug in the nanoparticles was determined using UV-Vis spectrophotometer at a wavelength of 280 nm. Freeze dried DAuNps were dissolved in distilled water under vigorous stirring for 30 min. The supernatant was obtained after centrifugation at 7000 g for 1 h. A standard calibration curve was made with the D.sod stock solution (dissolved in distilled water) covering a range of 0.1 mM-0.5 mM. To calculate the encapsulation effienceny and drug loading, the following equations were used:

$$\textit{Encapsulation Efficiency (\%)} = \frac{(\textit{Total drug} - \textit{Free unentrapped drug})}{\textit{Total drug}} * 100$$

$$\textit{Drug Loading (\%)} = \frac{\textit{Weight of drug in nanoparticles}}{\textit{Total weight of nanoparticles}} * 100$$

3.7 In vitro release study

Freeze dried DAuNps solution (2 ml) was suspended in 8 ml of phosphate buffer saline (PBS) solution pH 7.4).The mixture was stirred in a waterbath at 100 rpm.2 ml of release medium were withdrawn at various time intervals (1 ,2, 4, 6, 8, 24 h) and replaced with equal volume of fresh PBS. They were centrifuged at 2000 g for 5 min. The drug concentration in release medium was analyzed by UV-Vis spectrophotometer at 280 nm.

3.8 In vitro anti-inflammatory activity

In order to evaluate the anti-inflammatory activity in-vitro, method known as HRBC was used (Singh, Patil, Pal, & Ahmad, 2012).Fresh whole blood of total (3 ml) volume drawn from healthy donors was added to the same volume of sterilized Alsever's solution followed by centrifugation at 1008 g. Alsever's solution is a anti-coagulant solution which is used to prevent the clotting of blood. It consists of sodium citrate (0.8%), dextrose (2.05%), sodium chloride (0.42%) and citric acid (0.055%). After centrifugation, packed red blood cells were separated.

Packed red blood cells were washed three times with isosaline solution to remove the plasma proteins. The volume of the blood was reconstituted with isosaline solution as 10% (v/v) suspension.0.5 ml of HRBC suspension, 2 ml of hyposaline and 1ml of phosphate buffer were mixed separately with test sample, standard and control. After incubation at 37°C for 30 min, assay mixtures were centrifuged at 1000 g. The supernatant was then separated and haemogolobin content was measured with the use of UV spectrophotometer at a wavelength of 560 nm. The percentage of hemolysis was measured by assuming that control produced optimal hemolysis i.e hundred percent efficacy.

% Protection =

$$100 - \frac{(OD\ sample)}{(OD\ control)} * 100$$

3.9 In vivo anti-inflammatory activity

Egg albumin induced paw edema method was used to assay the in-vivo anti-inflammatory activity (Anosike, Obidoa, & Ezeanyika, 2012).Four groups of BALB/c mice were created which included 5 mice each. 0.1ml of fresh egg albumin (undiluted) was injected to the left hind paw of mice to induce paw edema (Figure 4 (a) and (b)).Paw thickness were calculated at 1, 2, 3, 4, 5 h using vernier caliper after the administration of phlogistic agents (Jung et al., 2015). Phlogistic agents are those agents which induces fever and inflammation.

(a) (b)

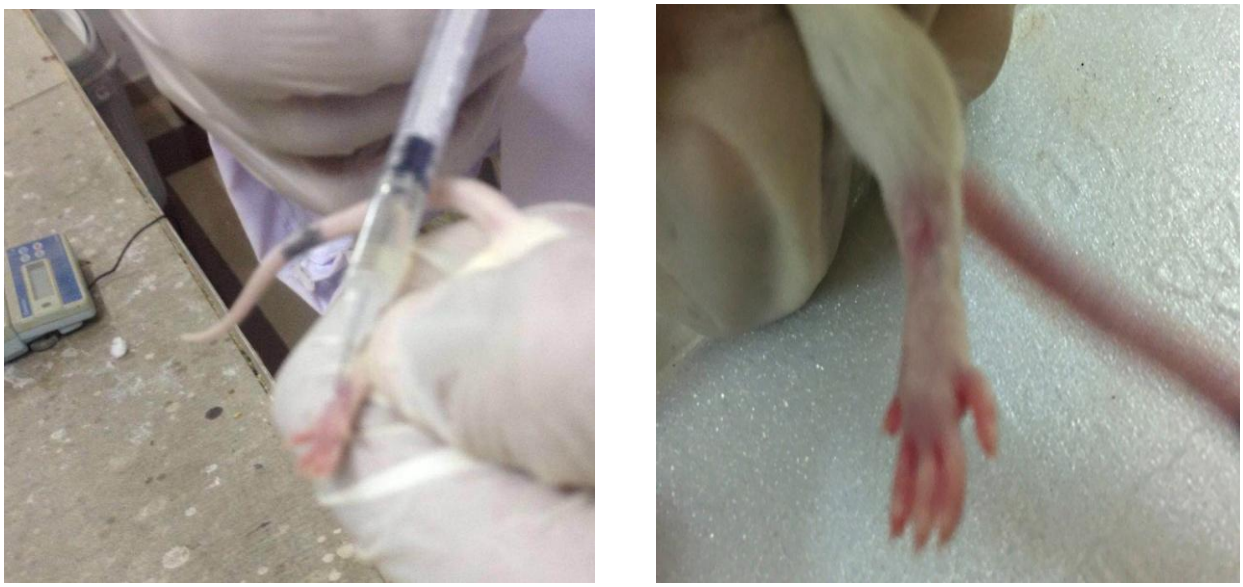

Figure 4: (a) Administration of fresh undiluted egg albumin to the left hind paw (b) Paw edema (swelling and redness)

CHAPTER 4: RESULTS

4.1 Biosynthesis and characterization of diclofenac sodium coated gold nanoparticles

Chemical reduction method was used to develop the D.sod gold nanoparticles**.** During the synthesis of D.sod gold nanoparticles, the purple colour was observed upon the addition of NaBH4 in a drop wise manner to the reaction mixture. In order to get the optimized DAuNps we have carried out a series of reactions using different concentrations of diclofenac sodium (Figure 5) at different temperatures (Figure 6) and pH (Figure 7). The optimum ratio, temperature and pH of diclofenac sodium coated gold nanoparticles were found to be 5:1:0.05 (D.sod:HAuCl4:NaBH4), 50ºC and pH 6.UV-Vis absorption spectroscopy of D.sod and DAuNps displayed Surface Plasmon Resonance (SPR) bands at about 302nm and 529nm respectively (Figure 8 (a) and (b)).

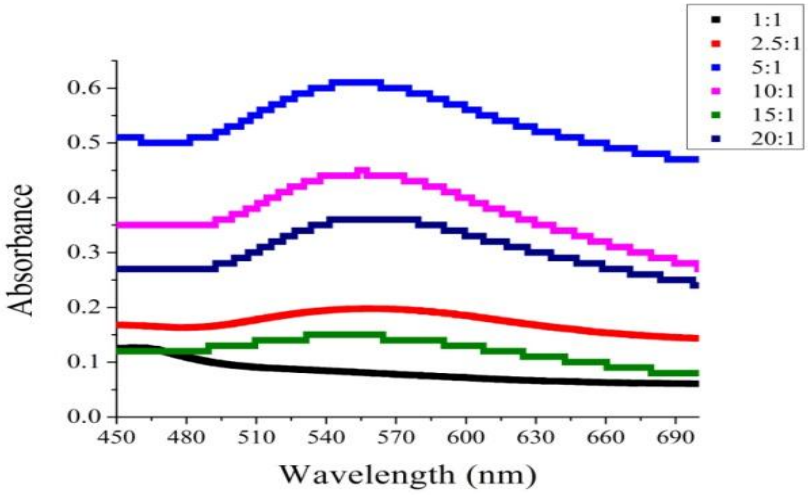

Figure 5: Optimizing the reaction by changing the concentration of diclofenac sodium

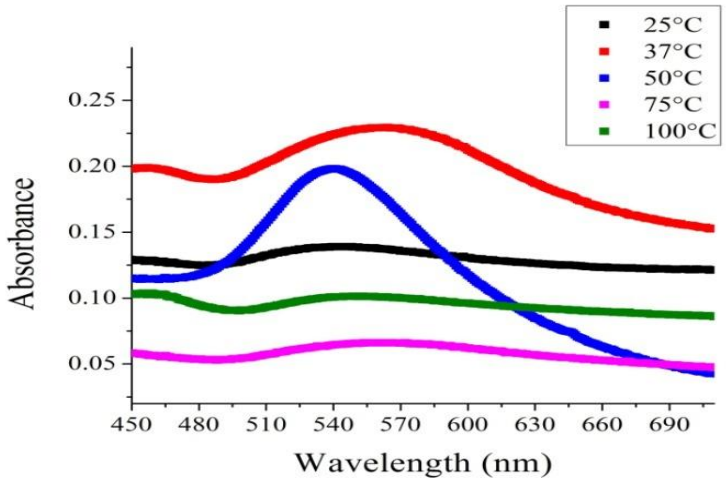

Figure 6: Optimizing the reaction by changing temperature

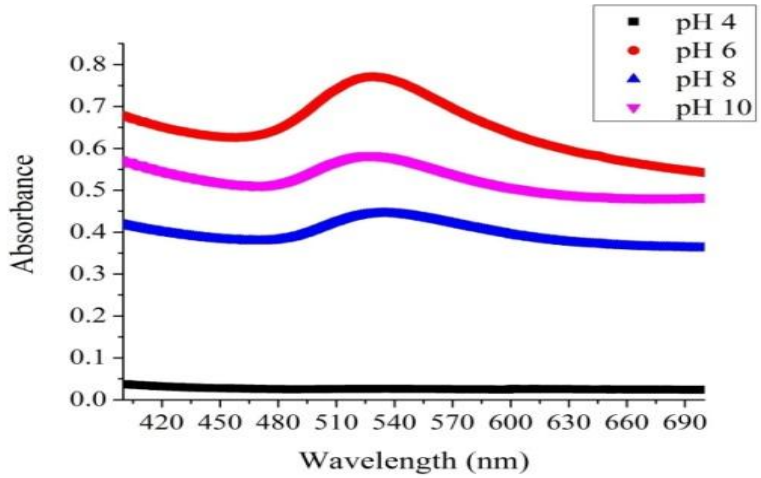

Figure 7: Optimizing the reaction by changing pH

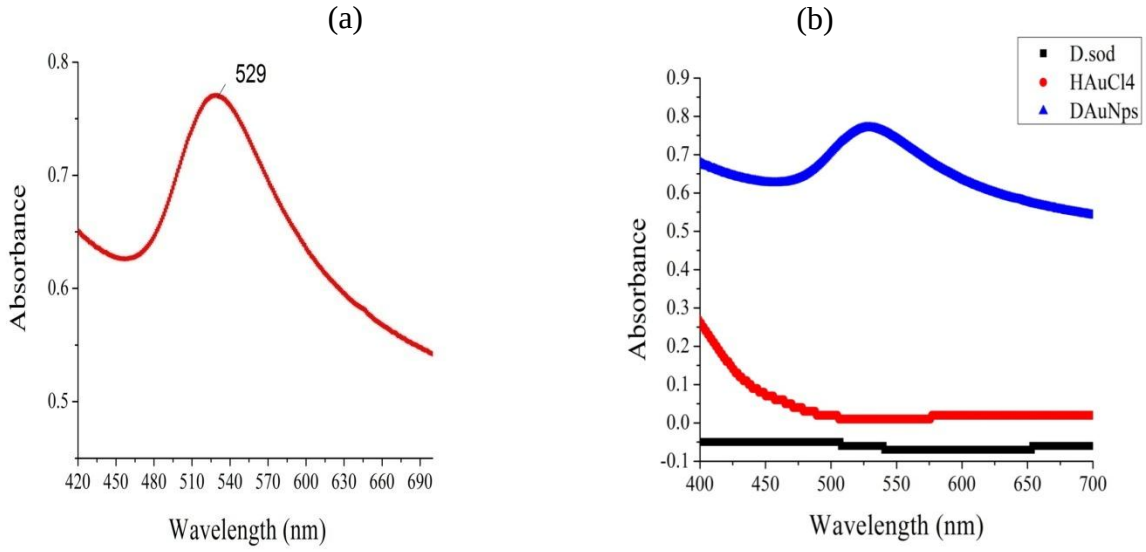

Figure 8: (a) UV-vis spectra of DAuNps (b) Comparative UV-vis spectrum of D.sod, HAuCl4 and DAuNps

4.2 FTIR spectroscopy analysis

Conjugation of diclofenac sodium with gold is supported by FTIR spectroscopy (Figure 9), where absorbance bands related to diclofenac sodium are observed in the region of 500-3500 cm^{-1}. The important diclofenac sodium spectrum bands appeared at different wave numbers such as 1552.52, 1452.15 and 743.93 cm^{-1} are present in the spectrum of DAuNps .It is evident from the figure the intensities of DAuNps were decreased which indicates that some specific groups are responsible for the reduction and stabilization of nanoparticles. Most probably the disappearance of –OH groups and the appearance of new peak at 1600 representing the aromatic groups (C=C) is responsible for the reduction of AuIII ions.

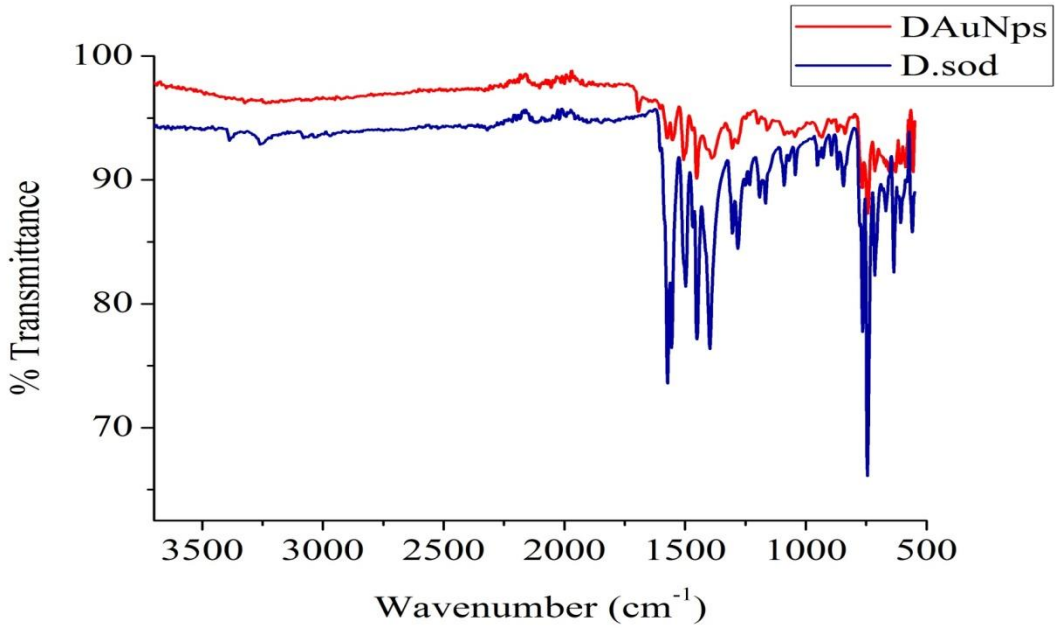

Figure 9: Comparative FTIR spectrum of D.sod and DAuNps

4.3 Scanning Electron Microscopy

Morphology of nanoparticles are determined by numerous microscopic techniques such as transmission electron microscope (TEM) and scanning electron microscopes (SEM).Scanning Electron Microscopy (SEM) is the most popular for nanoparticle analysis.SEM analysis was used to investigate the shape, morphology and size of DAuNps. The spherical structure of PEG-DAuNps was confirmed by Scanning Electron Microscopy (Figure 10)**.** The average size of prepared nanoparticles was 26nm.

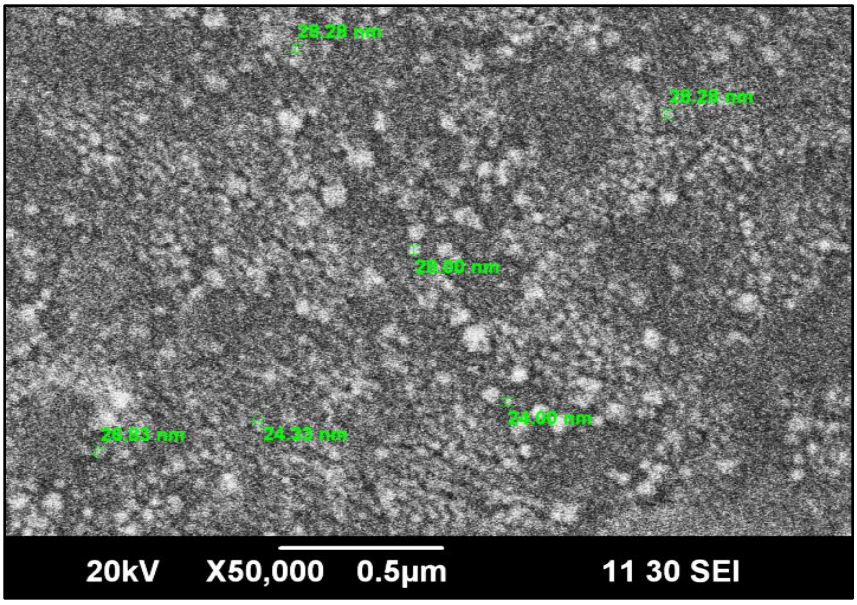

Figure 10: SEM micrograph of DAuNps

4.4 Drug encapsulation efficiency

Calibration curve of D.sod stock solution was plotted (Figure 11). The drug entrapped into nanoparticles was 18.84% and loading capacity was 32.41%.

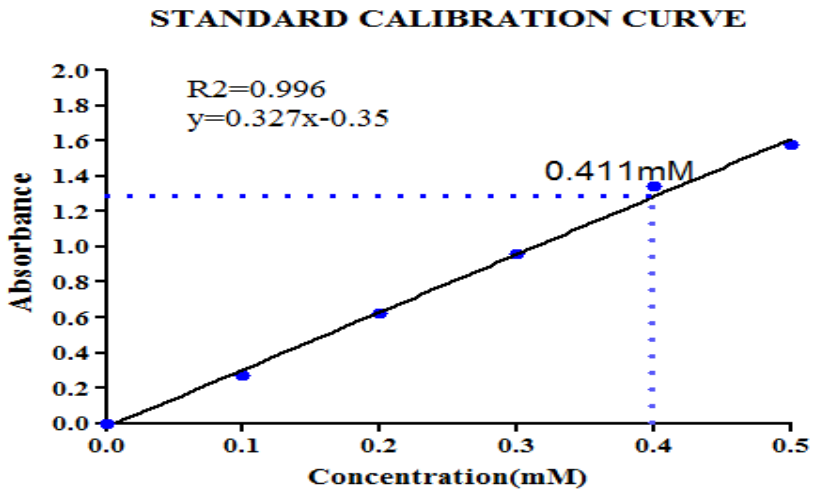

Figure 11: Calibration curve of Diclofenac sodium

4.5 In-vitro release study

Figure 12 shows an initial burst release of D.sod from nanoparticles due to the presence of D.sod on the surface of nanoparticles. Rapid leakage of D.sod from nanoparticles was ended very early and after some time sustained drug release was achieved. An initial fast release pattern is beneficial in terms of analgesic activity as it helps to achieve the therapeutic concentration of drug in minimal time followed by slow release to maintain, sustain and controlled release effect.

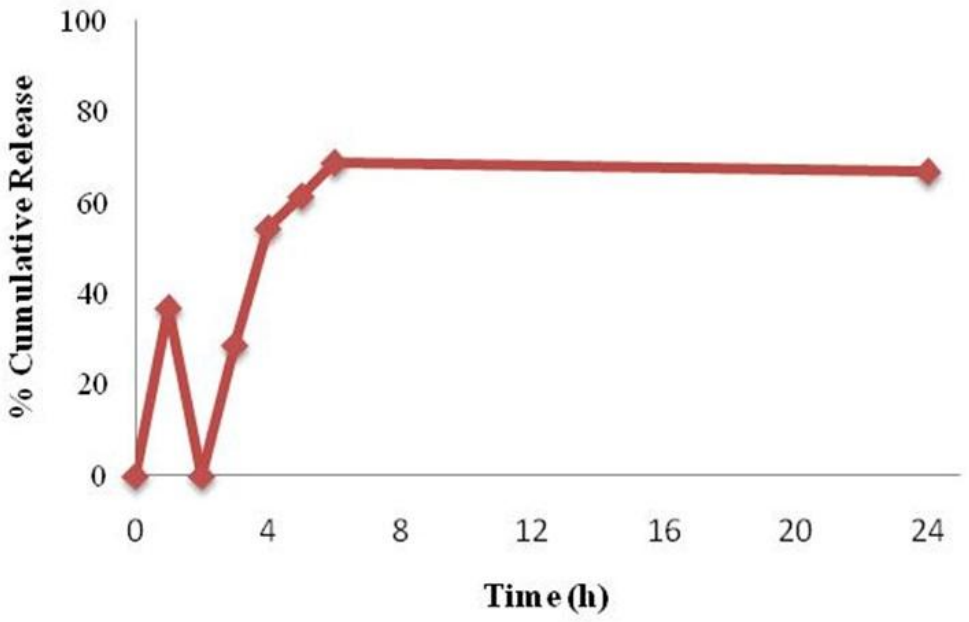

Figure 12: Cummulative drug release of Diclofenac sodium

4.6 In-vitro Anti-inflammatory activity

The membrane stabilizing activity of D.sod and PEG-DAuNPs were tested. PEG-DAuNps showed higher stabilization towards HRBC membranes when compared with D.sod (Figure 13). HRBC method was chosen due to the similarity of erythrocyte membranes to the lysosomal membranes. The stabilization of HRBC membrane is important by preventing the release of proteases and bactericidal enzymes, which cause inflammation.

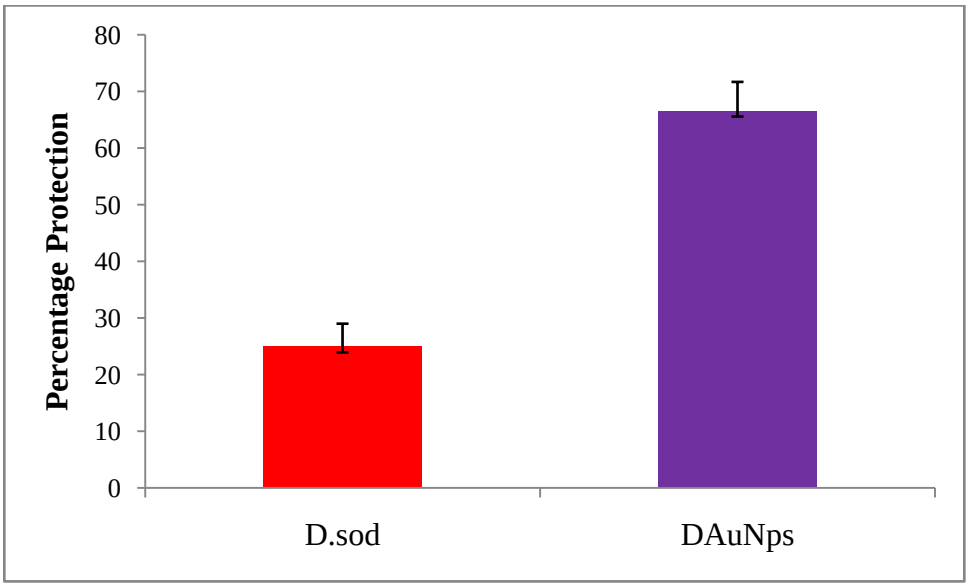

Figure 13: Percentage protection of D.sod and DAuNps

4.7 In-vivo anti-inflammatory activity

The PEG-DAuNps treated groups showed faster reduction in the paw thickness of mice as compared to D.sod and untreated groups (Figure 14).The synergistic action of inflammatory mediators such as serotonin, histamine and bradykinin at the inflammation site induces edema which results in an increased vascular permeability and blood flow. The administration of the phlogistic agent induces edema at early phase due to the release of serotonin and histamine which lasts for 2h and at late phase of edema the bradykinin, protease, prostaglandins and lysosome releases. The PEG-DAuNPS reduces the vascular permeability and fluid exudation which further suppresses the paw edema by preventing the release of inflammatory mediators such as histamine and 5HT in the paw of mice.

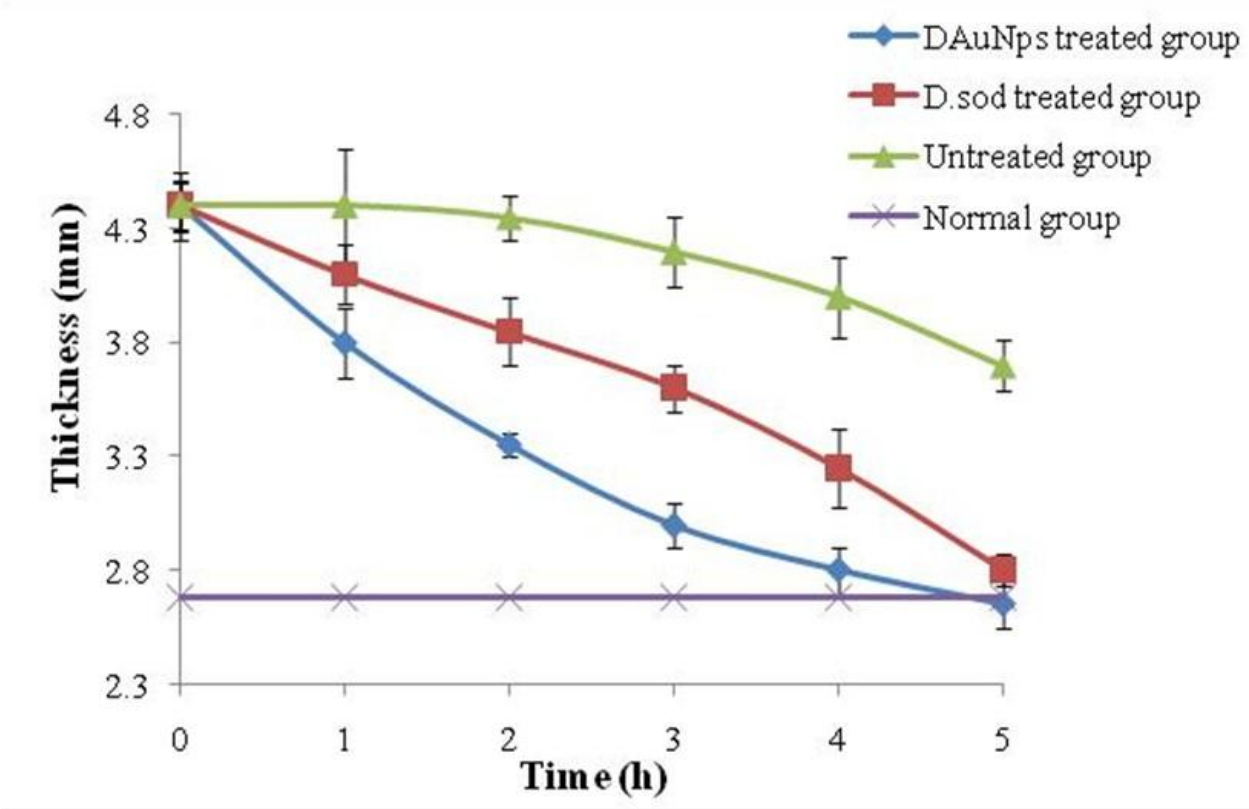

Figure 14: Time based recovery inflammation with DAuNps, D.sod, Untreated and Normal groups.

CHAPTER 5: DISCUSSION

Inflammatory diseases such as rheumatoid arthritis, osteoarthritis, autoimmune diseases etc are currently treated with non-sterodial anti-inflammatory drugs (NSAIDS).NSAIDS usually work by preventing the prostaglandins synthesis which causes inflammation. Diclofenac sodium is a well known NSAID used in the treatment of rheumatoid arthritis, osteoarthritis and ankolysing spondylitis. Diclofenac sodium competitively inhibits the prostaglandin (PGE2) by suppressing the expression of cyclooxygenase enzymes such as COX-1 and COX-2. Due to short half life of diclofenac sodium, it is usually given at high doses which cause severe adverse effects.There are various side effects related to the diclofenac sodium use such as gastrointestinal complications, renal failure, liver injury and myocardial infarction.

Nanoparticles based drug delivery systems are more potential nanocarriers because of having various benefits such as desired targeted delivery, modified drug release profile and increased drug stability. Gold nanoparticles are promising nanocarriers for anti-inflammatory drugs. They are less toxic and highly stable. They easily interact with the surface of molecules. Gold salts were used in the treatment of rheumatoid arthritis. They suppress the TNF-α, IL-2, IL-17 levels expression to inhibit inflammation. They also inhibit the reactive oxygen species to prevent the inflammation process.

In the present study, pegylated diclofenac sodium gold nanoparticles have been formulated. Purple colour of synthesized diclofenac sodium gold nanoparticles has been observed. Optimization of various parameters such as drug concentration, pH and temperature for the synthesis of diclofenac sodium coated gold nanoparticles were achieved. The optimized ratio, temperature and pH was found to be 5:1, 50ºC and pH 6.The diclofenac sodium gold nanoparticles was almost consists of monodispersed spherical DAuNps. The conjugation of diclofenac sodium with gold was confirmed by FTIR. The FTIR anlaysis also suggested that diclofenac sodium is properly loaded into the DAuNps.The drug encapsulation effiencey was determined. Drug release profile study suggests that linear curve was obtained which indicates that there is a sustained availibilty of drug in the desired organ.The physiological activity of the drug was achieved with the lower dose of formulations. HRBC method was selected for the evaluation of in vitro anti-inflammatory activity.

In vitro results indicated that PEG-DAuNps had higher protection towards human red blood cell membranes as compared to diclofenac sodium because it stabilizes them by preventing the lysosomal enzymes release.In vivo results showed that PEG-DAuNps reduced paw edema faster than diclofenac sodium because it suppresses the inflammatory mediators such as histamine and 5HT.As pegylated diclofenac sodium coated gold nanoparticles have multifunctional properties, it could be used as a promising therapeutic agent with reduced side effects for the treatment of acute and chronic inflammatory diseases.

CHAPTER 6: CONCLUSION

Since D.sod is known to have low bioavailability and is toxic in excess therefore its nanoparticles were prepared which proved to have higher bioavailability with no toxicity even at higher concentrations. Characterization data suggests that the new formulation presents suitable properties in terms of size, stability properties regarding to its potential to treat targeted inflammatory diseases. The results presented within this work are a first stage to the development of a new formulations platform suitable for target delivery of drugs intended by different pathologies. The optimum particle size indicates that the formulations were quite stable with satisfactory drug release. The efficiency of drug loading was very satisfactory employing the simple physical adsorption method. This system holds the potential of being applicable to other drugs as a reference delivery platform in terms of enhancing patience compliance with less dose interval and also eliminating systemic side effects. It is concluded from the study that as no obvious side effects have been observed with diclofenac sodium gold nanoparticles treatment, it can be claimed to be a better alternate to the mere drug.

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