CHAPTER 4

PLANT POLYPHENOLS IN CROSSLINKING CARTILAGE COLLAGEN AND DEVELOPMENT OF DEPOT DRUG DELIVERY SYSTEMS FOR CARTILAGE THERAPEUTICS

4.1 INTRODUCTION

4.1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease, characterized by inflammation of the synovium, synovial hyperplasia and infiltration of inflammatory cells leading to pannus formation and irreversible articular cartilage (AC) and bone destruction. The inflammatory process primarily affects the lining of the joints (synovial membrane). The inflamed synovium (synovitis) leads to erosions of the cartilage and bone and sometimes leads to joint deformity. RA is associated with fatigue and prolonged stiffness after rest. Pain, swelling, and redness are common joint manifestations. It is more common in females than males and can affect all ages, including children (Still’s disease), although it usually develops between the ages of 35 and 55 years. The cause of RA is not known. Yet, new research is giving us a better idea of what makes the immune system attack the body and create inflammation. It is a systemic disorder in which inflammatory changes affect not only joints but also many other sites including the heart, blood vessels and skin (Strietholt et al 2008, Jean-Gilles et al 2012).
Distinct mechanisms regulate inflammation and matrix destruction, including damage to bone and cartilage. Given the heterogeneous response to therapy, it is clear that RA is not just a single disease; instead many pathways can lead to auto reactivity with similar clinical presentations (Lee & Weinblatt 2001, Scott et al 2010). In RA, immune cells release inflammation-causing chemicals. These chemicals can damage articular cartilage (AC) (the tissue that cushions between joints) and bone. Genes that affect the immune system may also make some people more prone to RA (Waugh & Grant 2014).

AC is responsible for frictionless movement between the articulating surfaces of knee. The thickness of the AC ranges from 1 to 5 mm, composed of chondrocytes embedded in a matrix made up of major components viz., type-II-collagen and proteoglycans (Mort & Billington 2001). Water comprises 60-80% of the wet weight of cartilage. Its permeation into the AC matrix plays an important role in supply of nutrition. In addition, it is an important medium for maintaining the bio-mechano properties of collagen (tensile strength) and proteoglycan (cushioning) of AC (Buckwalter & Mankin 1998). In arthritis, the destruction of the cartilage is associated with a reduced synthesis of the matrix components by articular chondrocytes and an enhanced breakdown of the matrix by proteolytic enzymes, mainly the matrix metalloproteinases (MMPs) (Strietholt et al 2008). Degradation of proteoglycan is an early and reversible process, whereas the breakdown of the collagen network by collagenases is believed to be irreversible (Mort & Billington 2001, Ulrich-Vinther et al 2003, L'Hermette et al 2006). Collagen α-chain cleavage results in loss of integrity of triple helix, which in turn results in destruction of fibril network. The enzymes capable of cleaving triple helical type II collagen are interstitial collagenase (MMP-1, or collagenase-1), neutrophil collagenase (MMP-8, or collagenase-2) and collagenase-3 (MMP-13) (Wu et al 1991).
Therapeutic treatments include administration of analgesics (for pain), NSAIDs and glucocorticoids (to reduce inflammation), DMARDs (to slowdown the progression of joint damage or disease) and currently genetically engineered biologics (to interrupt the cascade of events that drive inflammation) through oral, parenteral or local (intra-articular) routes (Tyyni & Karlsson 2000, Scott et al 2010). In addition, all anti-rheumatic and immune-modulating drugs (biological therapies) developed so far carry a high cost, considerable risk of adverse effects, some of which can be severe or even life threatening (Scott et al 2010). Therapies are offered in different combinations and at different stages of the disease aiming at complete remission from inflammation and swelling by blocking any one of the prime inflammatory process or collagenase activity (Tyyni & Karlsson 2000). However, this goal is only achieved in a small proportion of patients, and partial remission and frequent relapses are a common problem. A significant number of patients still do not respond at all to available treatments (Tyyni & Karlsson 2000).

In Indian medicine as well as other systems of traditional medicine, plant polyphenols are an integral part of the composition used for the treatment of arthritis (Pandey & Rizvi 2009). The plant polyphenolic compounds are abundant micronutrients in our diet with an average consumption of 1g day\(^{-1}\) (Manach et al 2004). Fruit skin, tea leaves and vegetables are the highest source of polyphenols and many are well known for their antioxidant activity (Pandey & Rizvi 2009). Epigallocatechin gallate (EGCG), quercetin (QUE), and catechin (CAT) are the major polyphenols in the preclinical research for the treatment of cancer (Makena & Chung 2007), arthritis (Adcocks et al 2002, Ahmed et al 2008), ageing (dal Belo et al 2009), diabetes (Loke et al 2008), cardio vascular diseases (Margina et al) and other inflammatory diseases. Tannic acid (TA) extracted from oak tree also known to have beneficial biological activities in cancer and diabetes treatment (Chen

Based on the conventional wisdom on vegetable tanning, we hypothesise that binding of polyphenols with type II collagen in AC can prevent the deterioration of cartilage. In vegetable tanning, type I collagenous matrices of animal hides and skins are preserved permanently by the use of tannins which are plant polyphenolic molecules of the category proanthocyanins and hydrolysable tannins (Madhan et al 2001, Madhan et al 2002). In addition to collagenase inhibiting properties, the binding of plant polyphenol with type II collagen will provide stabilization against collagenase action which would protect AC from further deterioration after the onset of arthritis. Here, we present experimental evidence to substantiate our hypothesis. The protection of AC through the binding of polyphenols with AC in addition to the well-established route in interrupting the signals of macrophage or inhibiting the MMPs is certainly expected to pave way for the lives of those who are impaired with rheumatoid arthritis disorders in controlling/alleviating further deterioration of AC.

Intra-articular drug delivery is the preferred standard for targeting pharmacologic treatment directly to joints to reduce undesirable side effects associated with systemic drug delivery (Betre et al 2006). The joint cavity constitutes a discrete anatomical compartment that allows for local drug
action after intra-articular injection, needed for the treatment of arthritic disorders and the relief of pain and inflammation. After oral or parenteral (i.v., i.m., s.c.) administration, the therapeutic agent is transported to the intra-articular site of action via the systemic circulation. The availability of drugs administered through oral route has very low chances to reach therapeutic concentration in the joint cavities and the drugs dissolved in the synovial fluid are rapidly eliminated from the joint ($t_{1/2}$ of about 0.1–6 h) (Day et al 1999).

In contrast, intra-articular (IA) injection of suitable drug delivery systems (DDSs) may enable the major part of the incorporated drug to be released in the vicinity of the target area. An obvious disadvantage of IA injections is the discomfort and pain they may cause and the possible risk of infection. There is a major challenge in IA drug delivery, considering that, based on the physiology of joint; residence times of IA injected drugs are very short due to rapid clearance of the drugs by the circulation. Drugs clearance is largely dependent on the size of the molecule. The intra-articular approach may be limited by the rapid clearance of the drugs from the synovial space resulting in only transient efficacy (Evans et al 2014).

Multiple IA injection causes non adherence and non-compliance to the therapy. Therefore, the number of IA injections per year should be reduced to a minimum. This emphasizes the need for the development of sustained release formulations, which support the continuous release of the drug from a depot in the joint space over a period of several weeks or months. Development of injectable depot formulation helps in the maintenance of therapeutic concentrations over extended periods. This depot devices offer an excellent alternative to multiple IA injections (Gerwin et al 2006, Butoescu et al 2009).

The most obvious advantage of this relatively simple form of injectable depot delivery is that only a minimum amount of drug is required to
exert the desired pharmacological activity and thus, minimizing drug exposure to inappropriate sites. Further, direct joint instillation may constitute the only realistic route of administration for chemical entities suffering from bioavailability problems and extensive degradation \textit{in vivo} (Okada et al. 1994).

Maintenance of therapeutic drug concentrations in the joint over extended periods of time can be achieved by repeated IA administrations or more ideally, by immobilization of the active agent in the form of an injectable depot formulation from which the drug is released in a controlled manner. E.g. long-lasting (weeks) corticosteroid suspensions constitute the only worldwide available depot formulation type for the IA route of administration. Such depot injectables have been used for more than three decades providing symptomatic relief of pain in rheumatoid arthritis (RA) and osteoarthritis (OA) (Beck et al. 1985).

In pharmaceutical product development, polymeric microparticles have been widely investigated as a carrier for IA drug delivery (Ratcliffe et al. 1984, Ratcliffe et al. 1987, Horisawa et al. 2002, Fernández-Carballido et al. 2004, Liggins et al. 2004, Thakkar et al. 2004a, Thakkar et al. 2004b, Saito et al. 2009). One of the technological resources used to improve the persistence of drugs at the site of action, in this case the joint cavity, is the use of therapeutical systems prepared from biodegradable and biocompatible polymers (Liang et al. 2004). Besides the need to design a biocompatible system, the release profile of the encapsulated compound needs to be reproducible and independent of the force of the joint. Erodible devices offer the advantage of their biodegradation, disappearing gradually while releasing the drug from the site of action. The use of microparticles composed of polycyanobutyl ester, gelatin and PLA elicited various levels of inflammation following their IA injection (Liggins et al. 2004).
Earlier study indicates that PLA and other such polymers mentioned above might not be suitable drug carriers for IA treatment of rheumatoid arthritis. Liggins et al (2004) have shown that the IA injection of paclitaxel loaded PLGA microspheres with a mean diameter of 50 μm had no adverse effect on the normal function of horse joints (Liggins et al 2004). In another study, complete drug release from PLGA microspheres was observed between 84 and 96 h (Fernández-Carballido et al 2004). Biodegradable polyester biomaterial polycaprolactone (PCL) have been very widely used because of their biodegradation characteristics and they are approved by the US-FDA. PCL is more stable and cheaper than the extensively used PLGA copolymers. Hence in this work, low molecular weight PCL has been chosen for the preparation of TA loaded microsphere. Microspheres prepared from low molecular weight PCL can reduce the time presence of microspheres and its associated debris after the complete release of the drug (Mitragotri & Yoo 2011, Dash & Konkimalla 2012).

In previous chapters the effect of crosslinking with or on collagen to bring about a change in the biomaterial characteristics were highlighted. This chapter focuses on the efforts of in situ crosslinking of type II collagen (in vivo) with polyphenol to protect the cartilage matrix against the degeneration in arthritic conditions. It is also planned to formulate and investigate polyphenol (proven to be efficacious) loaded PCL (MW ~14,000) microspheres using multiple (W/O/W) emulsion or double emulsion solvent evaporation method to deliver the polyphenol in the joint cavity for prolonged period to manage/inhibit effectively, the inflammation and other associated symptoms or progression of cartilage degradation associated with rheumatoid arthritis. Henceforth, the frequency of IA injection can be reduced thereby improving the patient compliance.
4.2 MATERIALS

Catechin hydrate (CAT), Quercetin dihydrate (QUE), Epigallocatechin gallate (EGCG), Tannic acid (TA), Collagenase (Type IA), Polycaprolactone (PCL, Mw ~14,000,) and Polyvinyl alcohol was purchased from Aldrich, and other analytical reagents used for this study were purchased from Sigma Aldrich, Bangalore.

4.3 METHODS

4.3.1 Articular Cartilage Explants

The fresh tibio-femoral joints of bovine were collected from the slaughter house. Bovine cartilage was removed from the tibial plateau and femoral condyles under aseptic conditions (Yates et al 2005) within 3 h after excision from the joint. Cartilage slices of thickness varying from 1.2 to 2.4 mm were dissected from the joint surface (lateral and medial condyles) using a sterile scalpel blade. Cartilage slices were punched mechanically to obtain cartilage samples of uniform size and weight. Cartilage samples were washed in cold saline, transferred to sterile double distilled water (cold) and stored at -40°C until use.

4.3.2 Estimation of Water Content

The cartilages were grouped into three as per weight uniformity and placed in 2 mL centrifuge tubes containing PBS. The grouped cartilages were utilized for the estimation of water, collagen and glycosaminoglycans (GAG).

Water content was estimated by analysing the weight difference between the wet cartilage and completely dried cartilage. Wet weight ($W_w$) of cartilage was determined just after careful drying of surface water using tissue paper. Then the cartilages are freeze dried to completely evaporate the water
using LARK Freeze Dryer, India. Finally the weight of completely dried cartilage was noted as dry weight ($W_d$).

The percentage of water content was derived using the Equation (4.1) given below:

$$
\%W = \frac{W_w - W_d}{W_d}
$$

(4.1)

Where, \(\%W\) = percentage of water content in the cartilage

\(W_w\) = weight of the wet cartilage

\(W_d\) = weight of the dry cartilage (after lyophilisation)

### 4.3.3 Estimation of Collagen Content

The collagen content was determined by estimating the hydroxyproline content of hydrolysed cartilage using dimethyl aminobenzaldehyde and chloramine T. Briefly, cartilages were treated with 6N HCl at 118°C for 12 h in sealed hydrolysis tubes. Then the hydrolysed samples were heated in a crucible to completely evaporate the HCl by repeated washing and heating process. After complete evaporation of HCl, the hydroxyl proline (HP) crystals were suitably diluted to standard volume of 5mL. This HP in samples were then oxidized by Chloramine-T to yield Pyrrole-2-carboxylic acid. This reaction was arrested with perchloric acid and the resulting product reacted with p- dimethyl amino benzaldehyde acid to give a colored complex which could be read at 557 nm on a spectrophotometer. A standard curve was generated with standard hydroxyproline at final concentrations of 2–20 µg. (Woessner 1961, Ishikawa et al 2004). The amount of collagen was calculated by multiplying the amount of hydroxyl-proline with conversion factor (CF) 7.4.
4.3.4 Estimation of Glycosaminoglycan (GAG) Content

Glycosaminoglycans (GAG) present in the cartilage explants were determined by papain digestion of the cartilage followed by the assay using dimethyl methylene blue (DMMB) dye (Farndale et al 1986, Adcocks et al 2002, Ahmed et al 2004b, Pearson et al 2010). Except hyaluronic acid, all GAGs possess N- and/or O-sulfate groups distributed on their disaccharide building blocks. Most sulfated GAG chains are covalently linked to core proteins to form proteoglycans (Bernfield et al 1999). Initially cartilage explants were incubated overnight at 65°C in 2 mL of papain digest solution i.e. Papain Solution (1%) which was prepared by dissolving 1g of papain in 100 mL PBE buffer [PBE buffer: 100 mM Na₂HPO₄, 10 mM EDTA, 5 mM cysteine, 500 mL deionised water, final pH 6.5] for complete digestion of cartilage. Then the digested sample was centrifuged at 6000 rpm to remove the insoluble components if any and the supernatant was stored at -4°C.

The total amount of GAG was estimated by adding 200 µL of DMMB solution (i.e. 25 mg of 1,9-DMMB was dissolved in 5 mL of ethanol and taken up to 1 L in 0.2% sodium formate buffer - pH 3.5) to the final volume of 50 µL of supernatant suitably diluted. A standard curve was generated with chondroitin sulphate (from shark cartilage) at final concentrations of 1–5 µg. The samples were read on a microplate reader at 525 nm.

4.3.5 Thermal Stability of AC Explants

Differential scanning calorimeter (DSC) analysis has been widely used to determine the physicochemical transformations that occur during thermal degradation. The thermal stability of cartilage depends on the distribution of therapeutic (polyphenol) molecules inside the matrix and its interactions with the collagen fibrils of AC. AC explants were treated with
polyphenols at concentrations of 200 µM for 48 h in a shaking incubator at 37°C. AC explants incubated only with PBS were used as controls. The treated cartilage samples were incubated in PBS for 24 h to wash out the excess polyphenols. After washing, the cartilage samples (with and without polyphenol treatment) were placed on tissue paper to remove excess surface buffer (the moisture of AC is ~65%), then weighed and placed in an aluminum pan for calorimetric analysis using TA Instruments Model DSC Q200. The samples were analyzed in the temperature range from 25°C to 250°C at a heating rate of 5°C per min (Than & Kereskai 2005, Csotye et al 2009).

4.3.6 Enzymatic Stability of AC Explants

To study the effect of polyphenol treatment on the enzymatic stability of AC, the explants were treated with 200 µM polyphenols (CAT, QUE, EGCG, and TA) prepared by using PBS. This treatment had been carried out for 48 h in a shaking incubator at 37°C. AC explants incubated only with PBS were used as controls. The treated explants were again incubated for 24 h in PBS to wash out free polyphenols, as their presence could inhibit collagenase. Then, the explants were incubated in collagenase at 37°C for 96 h. The ratio of collagen (in cartilage explants) to collagenase was maintained at 50:1 (w/w), and the reaction was buffered at pH 7.4 with 0.1 M Tris-HCl and 0.05 M CaCl₂. After 96 h, the reaction was stopped, and the mixture was centrifuged for 15 min at 10,000 rpm. The supernatant was analyzed for soluble collagen and GAGs using hydroxyl proline assay using woessner method (Woessner 1961) and DMMB dye (Ahmed et al 2004b, Pearson et al 2010) respectively, and the percentage release thereof was then calculated.
4.3.7 Physical Stability of AC Explants

To study the effect of the polyphenols on the compression properties of cartilage, the compression strength of the cartilage with and without incubation in polyphenols were determined using a Brookfield CT3 10K Texture Analyzer, USA (shown in Figure 4.1). About 15 pieces of cartilage explants with 5 mm in diameter within a narrow weight range were obtained. The cartilage samples were made into 5 groups (3 samples per group). Four cartilage groups were incubated in 200 µM (37°C) polyphenols viz., CAT, QUE, EGCG and TA respectively for 96 h. Control group of cartilage was incubated in PBS and stored at 4°C. The cartilage samples prior to compression measurement were equilibrated with PBS pH 7.4 for 4 h and then placed directly below the probe in a stainless steel plate, with a thin layer of PBS on the circular plate of the instrument. The probe was allowed to compress the cartilage with the target of 50% compression at a defined speed (0.1 mm/sec) to examine the unconfined compression properties of the cartilage (Barker & Seedhom 2001, Ahmed et al 2009).

Figure 4.1 Brookfield texture analyser used for compression analysis of articular cartilage
4.3.8 In vivo Studies in Collagen Induced Arthritic Rats

4.3.8.1 Animals and grouping

Forty two, female wistar rats, 6 - 8 week old, weighing in the range of 130-180 g were used to evaluate the effect of intra-articular injection of polyphenols (EGCG and TA) in protecting AC during arthritic conditions. All the rats were purchased (after the approval of the institutional animal ethical committee (IAEC)) from the National Centre for Laboratory Animal Sciences (NCLAS) - National Institute of Nutrition (NIN), Hyderabad. The rats were housed under the norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in the institutional animal house, grouped, fed a standard commercial diet with tap water ad libitum. The animals were grouped for both prophylactic and therapeutic treatment conditions as follows:

Negative Control

Group NC - 6 rats

Prophylactic Treatment

Group PPC (Positive Control) - 6 rats
Group PE (EGCG) - 6 rats
Group PT (TA) - 6 rats

Therapeutic Treatment

Group TPC (Positive Control) - 6 rats
Group TE (EGCG) - 6 rats
Group TT (TA) - 6 rats
4.3.8.2 Induction of Collagen Induced Arthritis (CIA)

a) Preparation of Heat Killed Mycobacterium: The heat killed Mycobacterium tuberculosis (HkMtbd) was collected from Tuberculosis Research Centre, Chetput, Chennai. HkMtbd cells were pelleted by centrifugation at 4000 rpm for 30 min. The pellet was mixed with 10 mL of organic mixture (5mL diethyl ether + 5mL ethanol) and kept for 60 min at room temperature. This process was repeated twice and final pellet was kept for air drying in cold room for 2-3 days (Kumar et al 2002, Ganesan et al 2008).

b) Preparation of Adjuvant: Type II collagen (CII) from bovine AC was prepared and purified (See Appendix 1), the final concentration of 2 mg mL$^{-1}$ was obtained by suitable dilution using 0.05 M acetic acid and stored at 4°C. On the day of CIA induction, HKMtb were added to a mixture of equal volume of CII solution and Complete Freund's Adjuvant (CFA) to get a final concentration of 2 mg mL$^{-1}$. The antigenic adjuvant mixture was emulsified at 12,000 rpm using Homogenizer (IKA – T25 Ultra Turrax, Germany) in an ice bath.

c) Collagen induced arthritis: CIA was induced in 36 rats except the negative control (i.e. without disease) by intradermal injection of antigenic mixture at the back side (dorsal part) and rear feet of the rats. The antigenic mixture (300 µL in 6 divided doses) was injected over four sites at the back of the rat and remaining two at each foot on day 0(Induction Injection) and similarly on day 7 (Booster Injection). The rats were examined periodically for arthritis development through paw volume measurements of left and right knees using plethysmometer.
4.3.9 Effect of Intra-articular Injection of EGCG and TA (Prophylactic and Therapeutic) in protection of AC of CIA rats

The EGCG or TA solutions were prepared freshly on the day of injection by dissolving the desired weights in sterile phosphate buffered saline (PBS) – pH 7.4 under aseptic conditions. For each leg, a dose of 300 µg (i.e. 25 µL from 12 mg mL\(^{-1}\) stock solution) of EGCG or TA was injected intra-articularly in the rear tibio-femoral joints of the rats belonging to the respective groups. In prophylactic groups, five doses (i.e. on day -13, -10, -7, -4, -1.) of EGCG and TA injection were completed before the first arthritis induction injection (i.e. day 0), whereas in therapeutic groups, the dosing of polyphenols was on day 19, 22, 25, 28 and 31.

The prophylactic positive control (PPC) and therapeutic positive control (TPC) were injected intra-articularly with sterile PBS (25 µL per leg). On the day of sacrifice, legs of rats (the tibio-femoral joints) were harvested, X-rayed and then immediately fixed in 10% buffered formalin, decalcified in 5% nitric acid, dehydrated and embedded in paraffin. Embedded sections of 3 µm thickness were stained with haematoxylin and eosin (H&E) (Morinobu et al 2008). The H&E staining of joints were carried out to observe the cartilage damage and assigned scores as follows:

- Score 0 (normal cartilage) - normal appearance
- Score 1 (minimal damage) - minor destruction of the cartilage surface
- Score 2 (moderate damage) - clear loss of cartilage
- Score 3 (maximum damage) - cartilage almost absent in the whole joint (Svelander et al 2009).
4.3.10  Development of Polyphenol Loaded Depot Systems

4.3.10.1  Preparation of polyphenol loaded polycaprolactone microspheres

Polyphenol loaded polycaprolactone microspheres were prepared by double emulsion solvent evaporation technique with the slight modification of the previously reported procedure(Natarajan et al 2011).

The selected polyphenol of 25 mg was made into drug solution by dissolving in 2.5 mL of water and emulsified in the organic phase prepared by dissolving the PCL of 500 mg in 10 mL of DCM with different concentration (i.e. 0, 5, 7.5, 10 and 12.5%) of span 80 at 15,000 rpm for 5 min using IKA T-25. This emulsion was added drop wise using 5 mL syringe (for 5 min) into 400 mL of 0.5% aqueous polyvinyl alcohol solution (in 500 mL beaker). The stirring was maintained for 5-6 h at 2500 rpm, leading to a total evaporation of the solvent. The microparticles were then recovered by filtration, and dried at room temperature for 12 h.

In total five formulations were prepared according to the preparation conditions mentioned above with varying concentration of span 80 and named as F1-F5.

4.3.10.2  Characterisation of microspheres

Appropriate assessment of an injectable dispersed system requires characterization of both chemical and physical stabilities. Physical properties are very important with respect to the performance of dispersed systems. TA release rates from microspheres are often dependent on particle size, as well as other factors such as polymer type and molecular weight.
a) **Particle size determination**: Particle size distribution is one of the most important characteristics of injectable dispersed systems. For example, sedimentation or creaming tendencies of a dispersed system can be minimized by changing the particle size of the system. The stability of injectable dispersed systems can also be conveniently monitored by measuring changes in particle size and size distribution. The biofate of some dispersed system dosage forms is dependent on their particle size distribution. For parenteral delivery system, the diameter of the microparticles should be less than 250 µm, ideally less than 125 µm, to allow injections with acceptable needle diameters. Microsphere particle size and particle size distribution were measured by a laser light scattering analyser (CILAS 1180). A suitable amount of dried microspheres, from each formulation was suspended in water and sonicated for 1 min before measurement. The resulting homogenized suspension was then analysed for the volume mean diameter and particle size distribution called polydispersity index (PDI). The PDI (microspheres of non uniform size distribution) was calculated by the following Equation (4.2)

\[
Polydispersity = \frac{(D_{0.8} - D_{0.1})}{D_{0.5}}
\]  

(4.2)

Where, \(D_{0.8}\), \(D_{0.5}\) and \(D_{0.1}\) are the particle diameters determined at the 80\(^{th}\), 50\(^{th}\) and 10\(^{th}\) percentile of undersized particles, respectively. High polydispersity index values indicated the high level of non uniformity and it was used to characterise the microspheres as monodisperse, homogeneous and heterogenous systems.

b) **Entrapment efficiency**: The polyphenol content of biodegradable microspheres was determined for all the formulations by dissolving 10 mg of microspheres in 200 µL of dichloromethane (DCM) taken in 2 mL eppendorf tubes and vortexed for 10 min. Then 1800 µL of ethanol was added to precipitate polycaprolactone (insoluble in ethanol) and
vortexed for another 5 min to extract the TA into ethanol. The resulting solution was centrifuged for 10 min at 3000 rpm to settle down the precipitated polymer. The 200 µL of supernatant was diluted to 3 mL using ethanol and the absorbance measured by UV-Vis spectrophotometry and equivalent concentration was determined using the calibration curve prepared using the same proportion of solvents. The percentage (%) entrapment efficiency (EE) of the blend microspheres were calculated as per the Equations (4.3), (4.4) and (4.5).

\[
\% \text{ Drug loading (DL)} = \frac{\text{Weight of tannic acid in microspheres}}{\text{Weight of microspheres}} \times 100
\]  
\[\text{(4.3)}\]

\[
\% \text{Theoretical Loading} = \frac{\text{Weight of tannic acid added}}{\text{Weight of (Tannic acid + polymer) added}} \times 100
\]  
\[\text{(4.4)}\]

\[
\% \text{Entrapment Efficiency (EE)} = \frac{\% \text{Drug Loading}}{\% \text{Theoretical Loading}} \times 100
\]  
\[\text{(4.5)}\]

c) **Optical and scanning electron microscopy**: The optimized formulation selected from above characterization was viewed through optical microscope for its morphology and its sphericity.

Scanning electron microscopy is an excellent tool for physical observation of morphological features, like size, shape and surface characteristics. The microspheres were sprinkled on to one side of adhesive stub. The stub was then coated with conductive gold with EMITECH SPUTTER COATER and was examined under TESCAN scanning electron microscope for qualitative assessment of morphology of microspheres.
d) **Drug - Excipient interaction and Polymorphism studies:** FTIR spectra were taken on to investigate the possible chemical interactions between the drug and the polymer in the microsphere formulation. Samples were crushed with Potassium bromide (KBr) to get the pellets. The spectra of polymer, drug loaded microspheres are recorded.

Thermal analyses of the microspheres and of the pure drug were performed with a differential scanning calorimeter (TA Instruments, model Q100 MDSC). Samples of 2.5–12 mg were placed into aluminium containers and heated, at a constant rate (5°C min$^{-1}$), from 20 to 250°C under nitrogen atmosphere.

The crystalline microstructure of PCL microparticles is expected to have impact on behaviour of drug release. The crystalline microstructure of polymers is known to be influenced by the processing condition. X-ray patterns were obtained using a SEIFERT model JSO-DEBYEFLEX-2002 with a Cu Kα radiation, θ-2θ powder diffractometer set for an angle range of 5–70° 2θ. The step size was 0.04° 2θ, and count times were of 1 s per step.

4.3.10.3 **In vitro release studies**

Polyphenol release from the selected microsphere formulation (F3) was determined using Phosphate buffered saline (PBS) pH 7.4 as the release medium at 37±1°C. Microspheres were suspended in 10 mL of the dissolution medium in a 15 mL glass screw capped tubes covered with aluminium foil to protect from light. The tubes were tumbled end-over-end at 30 rpm in a thermostatically controlled oven. At regular time intervals, the tubes were centrifuged at 1500 rpm for 10 min and aliquot of the supernatant was collected and the concentration of polyphenol in the release medium was measured by UV-Vis spectrophotometer. The same volume of fresh PBS was added in order to maintain the sink conditions.
4.3.10.4 Collagen coating on polycaprolactone microspheres

In order to improve the biocompatibility of PCL, and to avoid any inflammation and foreign body response, simple collagen coating process were carried out to mimic the particles as ECM. Initially 5 mL collagen solution (3 mg mL\(^{-1}\)) was taken in the 15 mL centrifuge tubes. The completely dried TA loaded PCL microspheres (50 mg) were added to each centrifuge tubes and kept undisturbed for 30 min. Then all the centrifuge tubes were centrifuged at 6000 rpm for 10 min to decant the supernatant. The settled microspheres were washed twice with distilled water to separate the coated particle. The collagen coated microspheres were dried at room temperature (37 ± 2ºC) and collected and further confirmed by a scanning electron microscope (JEOL-JFC 6360 SEM, Japan) for the presence of collagen on the surface of the PCL microspheres.

4.4 RESULTS AND DISCUSSION

4.4.1 Water, Collagen, and GAG Estimation of AC Explants

In rheumatoid arthritis and osteoarthritis, the degradation of proteoglycans and collagen on the cartilage surface allows more water to penetrate and perturbs the organizational structure of the matrix. This in turn affects the elasticity and the load-bearing capacity of the cartilage. The cartilage explants were analyzed for water content, which was found to be 70.21%, in line with previous reports (Bhosale & Richardson 2008). The total collagen content of the wet cartilage used for the study was found to be 14.03%, which was about 47% of the dry weight. The GAG content of the explants used for the study was determined to be about 5.24% (on a wet weight basis).
4.4.2 Thermal Stability of Polyphenol-Treated Cartilage

Relatively little information is available on the thermal properties of mammalian hyaline cartilage, particularly the effect of drugs on thermophysiochemical properties of AC. Here, for the first time, bovine AC has been studied using DSC to evaluate the effect of polyphenols on the thermal stability of articular cartilage. DSC thermograms of native and polyphenol-treated AC are shown in Figure 4.2. Native (untreated) cartilage explants resulted in an endothermic peak at 91.26ºC due to the thermal denaturation of collagen in the AC. The polyphenol treated cartilages showed an increase in the thermal stability of collagen in cartilage by 10 to 15ºC; EGCG (105.06ºC) and TA (106.64ºC) showed the maximum increases in the thermal stability of cartilage compared to QUE (101.69ºC) and CAT (101.02ºC). The enhancement in thermal stability showed the presence of the binding of polyphenols with the collagen in the cartilage matrix. This indicated that polyphenol crosslinking with collagen.

Supra-molecular assemblies of type II collagen (CII) are similar to those of type I, forming fibrillar structures. AC, composed predominantly of CII, is a porous matrix that facilitates the diffusion of small molecules through the porous cartilage matrix, which could crosslink with the side chain functional groups of collagen. We show that treatment with polyphenols increases the thermal stability of articular cartilage. Of the various polyphenols tested, EGCG and TA showed a greater effect on thermal stability. Collagen is an inside-out protein where the side-chain functional groups are projected outward and the polyphenols, having multiple hydroxyl functional groups, can be involved in multiple hydrogen-bonded interactions with collagen, thereby conferring stability to the cartilage matrix. It has been proven that plant polyphenolic molecules can stabilize type I collagenous matrices through hydrogen bonding and hydrophobic interactions (Madhan...
et al 2005). Schlebusch & Kern (1972) studied the possible stabilizing effects of catechin on collagen of vascular tissue and found enzymatically and chemically more stable in in vitro and in vivo conditions.

Figure 4.2  Differential Scanning Calorimetric (DSC) thermograms of control (native) and polyphenols (catechin-CAT, quercetin - QUE, epigallocatechin gallate – EGCG and tannic acid - TA treated bovine articular cartilage samples

4.4.3 Enzymatic Stability of Cartilage Explants

Polyphenol binding and crosslinking render cartilage resistant to enzymatic degradation. The percentages of collagen and GAG released from the cartilage explants after the enzymatic treatment are presented in Table 4.1. The untreated cartilage showed collagen degradation of about 72 %, whereas the polyphenol-treated cartilage samples showed a significant level of protection from enzymatic collagen degradation. The EGCG, TA, and CAT treatments were more protective (i.e., statistically significant), showing only 24, 29, and 32 % collagen degradation, respectively, against collagenase attack. QUE showed lesser protective action against collagenolytic degradation. GAG release from the cartilage matrix after collagenase treatment was also lower for cartilage pre-treated with EGCG
(9.10 %) and TA (8.9 %) than the control, which was 18.8%; whereas, cartilage treated with CAT and QUE showed 13.1 and 12.8 % GAG release (Table 4.1).

**Table 4.1 Collagen degradation and release of GAGs from bovine articular cartilage (treated with or without polyphenols) against collagenase.** Data were analyzed using one-way ANOVA with Bonferroni post hoc test. The EGCG (P<0.01), TA (P<0.05), and CAT (P<0.05) treated groups showed significantly reduced percent degradation of collagen compared to controls. Similarly, the percent release of GAG in the EGCG- (P<0.01) and TA-treated (P<0.01) groups had significantly reduced (P-value: 0.0268) compared to controls but no statistical significance was observed in CAT and QUE. The values are represented as Mean ± SEM, n=3. (* indicates significant difference in comparison to control, P<0.05.)

<table>
<thead>
<tr>
<th>Polyphenol (200 µM)</th>
<th>% Degradation of collagen (from AC)</th>
<th>% Release of GAGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS medium)</td>
<td>72.5 ± 13.6</td>
<td>18.86 ± 0.60</td>
</tr>
<tr>
<td>Catechin</td>
<td>32.1 ± 6.0*</td>
<td>13.18 ± 1.43</td>
</tr>
<tr>
<td>Quercetin</td>
<td>42.7 ± 4.9</td>
<td>12.89 ± 1.19</td>
</tr>
<tr>
<td>Epigallocatechin gallate</td>
<td>24.6 ± 1.3*</td>
<td>9.10 ± 1.59*</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>29.0 ± 3.4*</td>
<td>8.96 ± 1.07*</td>
</tr>
</tbody>
</table>

This is consistent with an increase in the thermal stability of AC caused by EGCG and TA. In addition to the collagenolytic degradation of AC, there was a significant release of GAGs from the enzyme-treated AC, which must have been due to the release of GAGs associated with the fragments of the collagen fibrils digested from the cartilage. Interestingly, both TA and EGCG treatment exhibited higher efficacy in stabilizing cartilage for collagen degradation and GAG release in comparison to QUE and CAT. This could be due to the ability of EGCG and TA to form better crosslinking with collagen and other matrix components.

Recently, a study to improve collagen-based biomaterial used for skin anti-ageing has been conducted using polyphenols, and it was revealed
that the polyphenols block the site at which collagen is cleaved by collagenase (Jackson et al 2010). Previous studies have reported collagenase inhibitory effects caused by polyphenols in cartilage tissues and reconstituted collagen (Ahmed et al 2004b, Rasheed et al 2009, Jackson et al 2010). Since the polyphenol-treated cartilage was thoroughly washed to leach out any free polyphenols, the possibility that collagenase was directly inhibited by polyphenols is unlikely. Hence, the resistance to collagenolytic degradation is predominantly due to increased crosslinks between polyphenols and CII in the cartilage.

4.4.4 Physical Stability of AC (Compression Analysis)

The ability to bear a load is an important physical property of the AC. The effect of polyphenolic interactions may alter the compressive properties of the cartilage. To determine the changes in compressive properties, the compression load of AC samples with and without polyphenol treatment were analyzed and cartilage samples incubated for 96 h are presented in Figure 4.3. The control cartilage incubated in PBS for 96 h exhibited a mean load of 315.83 g for 50% compression of the AC. Though the mean values of polyphenol-treated ACs were slightly different in comparison to controls, the differences were not statistically significant.

As shown in Figure 4.3, polyphenol-treated cartilage explants showed less deterioration in load-bearing ability in comparison to controls. The change in load after polyphenol treatment was not more than 10% compared to the change in untreated cartilage (controls). This shows that polyphenolic treatment did not have a major impact on the change in the compression properties of cartilage compared to the untreated cartilage explants.
Figure 4.3  Compression load @ 50% compression of articular cartilage incubated for 2, 4, 6, and 8 days with or without polyphenols of 200 µM viz., CAT, QUE, EGCG, TA was used for the incubation in PBS. Figure shows 4 day results. No significant difference (P>0.05) was observed between the control and polyphenol treated samples. The values are represented in Mean ± SEM, n = 3

4.4.5  Effect of Polyphenols (EGCG and TA) in AC Protection in CIA Rats

*In vitro* studies clearly indicated that the treatment of cartilage with polyphenols stabilized the cartilage enzymatically, thermally, and mechanically. Cartilage treated with EGCG and TA showed better enzymatic stability in comparison to cartilage treated with CAT and QUE. Hence, EGCG and TA were further chosen to study the *in vivo* protective effect on cartilage degradation. We evaluated prophylactic and therapeutic strategies by intra-articular injection in a CIA rat model. Before adjuvant preparation, the purity of type II collagen was confirmed by gel electrophoresis and circular dichroism. The presence of single $\alpha_1$(II) band in the gel plate shows its homotrimeric helix as well as the purity of the collagen prepared for adjuvant preparation. The nature of collagen was also confirmed by CD studies (see
Appendix 1). A positive peak at 221 nm and a negative peak at 197 nm clearly demonstrate its presence as triple helix.

Because of the poor bioavailability of polyphenols in circulation, particularly in synovial fluids, it is likely that limited observations have been made concerning its salutary effect on cartilage. We hypothesize that polyphenols injected intra-articularly will interact with CII and stabilize against collagenolytic degradation. Furthermore, the more efficient (in the enzymatic stabilization of AC) polyphenols, viz. EGCG and TA, were selected for the in vivo studies using CIA rat models.

4.4.5.1 Prophylactic treatment

In prophylactic treatment groups, the paw volume stayed unchanged until day 8 (Figure 4.4). On day 15, we observed a significant (P<0.001, n=6) increase in paw volume in PPC, PT, and PE compared to NC, indicating the onset of CIA. There was no significant difference in paw volume at the onset of CIA between the PPC and the PT or PE groups. On the beginning of day 22, the PE rats were found to have a significant difference (increase) in paw volume until day 36 compared to that of PPC, and it was not observed in the PT treated groups. A significant maximum increase in paw volume was observed on day 29 (i.e. to 2.78 mL compared to that of positive controls, which had a volume of 2.21 mL). The prophylactic treatment of both polyphenols did not show any improvement in alleviating the inflammation in the paws.

The X-ray analysis of joints at the day of harvesting the tibio-femoral joints for histology was carried out to profile the cartilage damage. The X-ray pictures shown in Figure 4.5 were analysed for cartilage damage and it was difficult to differentiate the cartilage in both the tibia and femoral ends. In addition, from the histological analysis, the cartilage damage was
found to have significant cartilage degradation, and the scoring levels of cartilage degradation are presented in Figure 4.6.

![Figure 4.4](image)

**Figure 4.4** The paw volume changes in prophylactically treated groups from day -18 to day 43 (values are analyzed using two-way ANOVA using Graph Pad prism. “****” indicates significant difference in comparison to negative control (NC) (P<0.001) and “#” indicates significant difference in comparison to prophylactic positive control (PPC) (### represents P<0.001 and # represents P<0.05). The values are represented in Mean ± SEM, n=12

The combined scoring for cartilage degradation in PT and PE was significantly lower than in PPC (P<0.05). The evaluation of histological sections of all the groups was done blindly for cartilage damage and synovial inflammation. The synovial membrane and cartilage in the NC group was normal. PPC exhibited synovial adhesions and fibrous fatty tissue, and the synovial membrane was found to be inflamed and hyperplastic (Figure 4.7), confirming the induction of CIA. The synovial membranes of PT showed more inflammation than PE, while the latter group had few eosinophilic infiltrations. Cartilage in PT showed some surface structural deterioration and rice bodies in the joint space.
Figure 4.5  X-Ray analysis of tibio-femoral joints for assessing cartilage degradation profile in a) Negative control, b) Positive control, c) EGCG treated and d) TA treated (Left and right column indicates prophylactic and therapeutic treatment group respectively)

Figure 4.6  Cartilage degradation scores from histomorphological sections of prophylactically treated groups. “***” indicates significant difference that of negative control (P<0.01). Difference between the PPC and the PT or PE groups are statistically significant indicated as “#” (P<0.05). The values are represented in Mean ± SEM, n=6
Figure 4.7 Effect of prophylactic treatment of polyphenols on collagen-induced arthritis (CIA). Hematoxylin and Eosin-stained sections of tibio-femoral joints of rats. NC indicates Negative Control i.e. normal joint (without any induction and treatment). Remaining illustrations are from collagen-induced arthritis (CIA) joints; PPC—Prophylactic Positive Control; PE—Prophylactic EGCG; and PT—Prophylactic TA.

4.4.5.2 Therapeutic treatment

In the therapeutic treatment groups, the paw volume started to change on day 17, confirming the induction of CIA (Figure 4.8) that was significant (P<0.001, n=6) in the TPC, TT, and TE groups compared to NC. Early on, paw volume was not significantly different in the TPC, TT, and TE groups. In the case of therapeutic EGCG treatment, paw volume significantly increased on day 22 (P<0.05), but on day 43 (final), it had dropped significantly (P<0.01) to a volume of 2.00 mL compared to positive controls (2.29 mL). On day 43, the TT group also showed a significant (P<0.001) decrease in paw volume compared to TPC (i.e. 1.93 mL for TA and 2.29 mL)
for positive controls) (Figure 4.8). There was no significant difference between TA and EGCG in reducing paw inflammation.

In the therapeutic groups, the cartilage damage scores of TA-treated (TT) and EGCG-treated (TE) cartilage were significantly lower (P< 0.05) than that of TPC (Figure 4.9). The damage scoring of TE was not significantly different from that of TT. Visually, the cartilage damage was very mild to negligible in most TT and TE samples (Figure 4.10). Synovial inflammation was found in both the TA- and EGCG-treated therapeutic groups. However, the severity in both TT and TE was lower than in TPC.

Figure 4.8  Paw volume changes in therapeutically treated rat groups from day 1 to day 43 (values analyzed using two-way ANOVA using Graph Pad prism. “***” indicates significant difference in comparison to negative control (NC) (P<0.001) and “#” indicates significant difference in comparison to therapeutic positive control (TPC) (### represents P<0.001, ## represents P<0.01 and represents P<0.05), The values are represented in Mean ± SEM, n=12)
Figure 4.9  Cartilage degradation scores from histomorphological sections of therapeutically treated groups. “**” indicates significant difference in comparison to Negative control (P<0.01). Differences between the TPC and the TT or TE groups are statistically significant indicated as “#” (P<0.05). The values are represented in Mean ± SEM, n=6).

Figure 4.10  Effect of therapeutic treatment of polyphenols on collagen-induced arthritis (CIA). Hematoxylin and Eosin-stained sections of tibio-femoral joints of rats. NC indicates Negative Control normal (without any induction and treatment) joint. Remaining illustrations are from collagen-induced arthritis (CIA) joints; TPC - Therapeutic Positive Control; TE - Therapeutic EGCG; and TT - Therapeutic TA.
The onset of CIA was clearly established with the increase in paw volumes in both prophylactic and therapeutic groups (Figure 4.4 and 4.8). The lack of a decrease in the paw volume of the prophylactic group (Figure 3.4) is a clear indication that there is little influence of polyphenols in controlling either the time of onset of arthritis or the maintenance of the level of inflammation as measured by paw volume, whereas in the case of the therapeutic groups, there was again no delay in the onset of arthritis or the maintenance of inflammation except some reduction in paw volume at the end of the experiment (day 43) (Figure 4.8) compared to positive controls. In all cases, the positive controls (immunized and PBS-injected) had significant inflammation compared to un-immunized control. Also, the prophylactic intra-articular administration of EGCG or TA showed a significant (P<0.05) protective effect on the degree of cartilage damage compared to positive controls (Figure 4.6 and Figure 4.7). This indicated that AC in polyphenol-injected joints became resistant to the subsequent induction of inflammation-induced degradation.

In the therapeutic polyphenol group, there was some reduction in paw volume, indicating a decrease in the degree of inflammation only during the latter part of CIA as compared to paw volume in positive controls. This may be consistent with the known anti-inflammatory effects of polyphenols. Therapeutic groups also showed the significant benefit of intra-articular injections of polyphenols in protecting cartilage from degradation during CIA (Figure 4.9 and Figure 4.10). These observations are statistically significant (P<0.05). EGCG showed a better cartilage-protecting effect than TA. This indicates that AC in therapeutic polyphenol-injected joints become resistant to concurrent inflammation-induced degradation compared to subsequent inflammation in the prophylactic groups. The protective effect on cartilage in the therapeutic groups could have been caused by the direct inhibition of MMPs. However, polyphenols in synovial fluid would have been rapidly cleared and metabolized, and little of it would be available to inhibit the
gradual degrading process of MMPs on cartilage. Another possibility for the protective effect on cartilage could be a reduction in the degree of inflammation in the joints, but this was observed only in the latter part of CIA and was not confirmed by histopathological observation of inflammation in the synovium. Based on our in vitro data and the observations in the prophylactic polyphenol treatment groups, we attribute predominantly the binding of polyphenols as the likely cause of protection against cartilage degradation in the therapeutic groups.

Previously, catechin (20 µM) was found to inhibit the degradation of bovine nasal and AC explants by inhibiting the chondrocyte catabolic response (Adcocks et al 2002). In another study, EGCG (100 µM and 200 µM) was shown to be effective in inhibiting the IL-1β-induced production of matrix-degrading enzymes (Ahmed et al 2004b). Recently, curcumin (1–25 µM) and quercetin (10–50 µM) were also found to have an effect in inhibiting the matrix-degrading enzymes (Lay et al 2012).

Human collagen type II in the cartilage matrix is a long-lived protein with an estimated half-life of >117 years (Verzijl et al 2000). Collagen maturation involves extensive crosslinking, and it is a strategy used in the maintenance of cartilage for the life of most humans. Based on the conventional wisdom on vegetable tanning, we have hypothesized that polyphenols could be used to crosslink cartilage collagen type II and make it resistant to degradation as shown in schematic form in Figure 4.11. In this study, we have provided proof of this principle. Intra-articular injections of polyphenols preventively as well as in the milieu of inflamed joints prevented cartilage degradation. It is also possible that intra-articular injections of polyphenols may make osteoarthritic cartilage resistant to degradation. Further research is warranted to study means of delivering polyphenols selectively into a joint cavity. Hence, we can see a unique novel role for
intra-articular injections of polyphenols in the therapeutic treatment of cartilage degradation.

![Image of collagen crosslinking](image)

**Figure 4.11** Crosslinking of collagen using polyphenols: A schematic representation on the mechanism of polyphenolic crosslinking of articular cartilage in alleviating its destruction during arthritic condition

### 4.4.6 Tannic Acid Loaded Polycaprolactone Microspheres as injectable Depot Systems

The *in vivo* trials using CIA rats demonstrated both TA and EGCG were found to be efficacious in the management of arthritis. However in clinical applications it is challenging task to have the drug bioavailable sustainably for the treatment of knee joints. Delivery of the drug through
microspheres through intra-articular route would be a feasible option for the sustained availability of the drug and for better patient compliance. PCL microspheres are TA has been selected as a model drug to formulate a suitable depot system for intra-articular injection towards the treatment of cartilage degradation (i.e. cartilage therapeutics).

Oil in water (O/W) emulsion system is the conventional system used in the preparation of microspheres using solvent evaporation method. A major problem with this technique is a poor encapsulation efficiency of water soluble and moderately water soluble compounds, which partition out from the organic dispersed phase into the aqueous continuous phase. Successful entrapment of drug within the microspheres is thus highly dependent on solubility of the drug in the aqueous phase. High solubility of TA has to be taken into account for preparing microspheres with good entrapment efficiency. Water-oil-water (W/O/W) double emulsion system had been chosen for the encapsulation of TA in PCL. The microspheres were prepared as per the procedure given in method section to overcome the problem of low encapsulation of water soluble drug prepared by conventional oil/water emulsion solvent evaporation method. To increase the stability of the primary W/O emulsion (i.e. aqueous drug solution in polymeric organic phase), the non-ionic surfactant span 80 (HLB of 4.3) had been selected, and the concentration was varied to study its effect on the entrapment efficiency of the TA. The organic phase acts as a barrier for the two aqueous phases. The primary emulsion was then emulsified into the 0.5% aqueous solution having PVA as stabilizer to produce a multiple W/O/W emulsion and PVA acts as stabilizer to prevent the diffusion of active material towards the external aqueous phase.
Table 4.2  Particle size analysis and entrapment efficiency of TA loaded PCL microparticulates

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Mean Particle Size (µm)</th>
<th>Polydispersity Index</th>
<th>Entrapment Efficiency (%± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (0% span 80)</td>
<td>50.01</td>
<td>1.0597</td>
<td>65.35±6.85</td>
</tr>
<tr>
<td>F2 (5% span 80)</td>
<td>33.47</td>
<td>1.0297</td>
<td>81.39±3.10</td>
</tr>
<tr>
<td>F3 (7.5% span 80)</td>
<td>42.98</td>
<td>0.6410</td>
<td>85.08±8.22</td>
</tr>
<tr>
<td>F4 (10% span 80)</td>
<td>52.74</td>
<td>1.0632</td>
<td>99.83±9.91</td>
</tr>
<tr>
<td>F5 (12.5% span 80)</td>
<td>119.35</td>
<td>5.0132</td>
<td>108.61±16.19</td>
</tr>
</tbody>
</table>

The results of the particles average mean diameter, polydispersity (PDI), and entrapment efficiency (EE) were listed in the Table 4.2 and the five different formulations F1 to F4 have shown particle size ranging between 33 and 53 µm), formulation F5 showed a very high average particle size of 119 µm. This could be due to the concentration of surfactant used which might have exceeded its level in primary emulsion and might have disturbed to form a stable secondary emulsion.

F5 also shows high PDI, which is an indication of highly heterogeneous formation of microspheres compared to other formulations. Formulation F3 showed to be more homogenous compared to others, an essential requisite for the depot particulates to ensure the uniform drug release and easier injectability.

The EE of the F3 is found to be 85% compared to 65% for F1 which was prepared without surfactant. This result evidenced that the 7.5% of
span 80 has given a better stable emulsion which can be re emulsified to prepare microspheres with high load of TA through secondary emulsion. It also evidenced that the increase in emulsifier concentration increased the EE of the drug through a formation of stable primary emulsion. The increase in emulsifier to 10% and 12.5% has shown a EE of 99% and 108% which is higher than that of previous formulations, may be due to the usage of excess surfactant than the essential. As previously mentioned, the excess surfactant has caused a disturbance in formation of secondary emulsion in F4 and F5, which was seen by increase in mean particle size to 52 and 119 µm respectively from 42 µm of F3. The formulation F3 has low PDI and high EE and hence was selected for further characterization and release studies.

4.4.7 Morphology of Microparticles

Optical microscopy photographs of F3 formulation prepared were shown in Figure 4.12a, and from the figures it is clearly seen that the microspheres formed are spherical and discrete. However the surface of the spheres were observed to be rough.

SEM analysis was carried out to observe the surface morphology of the particulates and was shown in Figure 4.12b. The micrograph shows that the particles are spherical and surface was rugged and not uniform. There were aggregated particulates around the surface of the spheres caused due to adherence of newly forming particulates on the surface of stable particulates. This would be due to the slower evaporation of the solvent from the external aqueous phase.
Figure 4.12  (a) Visualised photograph of F3 through Optical microscopy, (b) Micrograph of F3 formulation through scanning electron microscope

4.4.8 FTIR Analysis

FTIR spectra obtained could confirm the chemical interactions in the microspheres. FTIR spectra of TA, PCL and TA loaded PCL microspheres are shown in Figure 4.13. The spectral examination of TA revealed that, broad peak maximum at 3328 cm\(^{-1}\) corresponds to the hydroxyl groups of TA. The vibrations assigned to aromatic rings are located between 1661 cm\(^{-1}\) and 1448 cm\(^{-1}\). FTIR spectrum of PCL showed prominent peaks at 1734 and 3430 cm\(^{-1}\), which correspond to the –CO (stretching) and –OH (bending) groups. The peaks at 2864 and 2948 cm\(^{-1}\) were related to the CH bond of saturated carbons. The TA loaded PCL microspheres were identical to those of PCL and appeared at almost the same wavenumber except in the hydroxyl region which had a slight increase in intensity between 3300-3400 cm\(^{-1}\), which could be due to the presence of TA. Considering low amount of TA in comparison to PCL in the TA loaded PCL microspheres, the molecular feature of PCL are more dominant in the TA loaded PCL
microspheres. This study confirmed the stability of the polymer in the processing conditions.

![FTIR Spectral Analysis](image)

**Figure 4.13** FTIR – spectral analysis of Polycaprolactone (PCL), Tannic acid (TA) and Tannic acid loaded microsphere (MSP)

### 4.4.9 DSC Analysis

DSC studies were performed to understand the nature of the encapsulated drug in the matrix. The physical state of TA in the polymer matrix would also influence its release characteristics. DSC analysis was
performed on plain TA, PCL, and TA-loaded PCL microspheres - F3 and the respective thermograms are shown in Figure 4.14.

Figure 4.14 Differential scanning calorimetric analysis of Polycaprolactone (PCL), Tannic acid (TA) and Tannic acid loaded microsphere (MSP)

Melting temperature ($T_m$) or endotherm peak of plain PCL and loaded microspheres were observed at 59°C. Melting temperature of tannic acid was observed at 85°C and it showed broad endothermic peak for dehydration from 102 to 110°C. These PCL microparticles showed a small
endothermic peak associated with TA at 210ºC suggesting that the drug was in the poly-caprolactone matrix. It is also estimated that the drug is dispersed in the amorphous region of the polymer matrix and not in the crystalline region.

4.4.10 Powder X-ray Diffraction (PXRD) analysis

PXRD of plain PCL, TA and TA loaded PCL microspheres (F3) are shown in Figures 4.15.

Figure 4.15 Powder X-Ray diffractometer analysis of Polycaprolactone (PCL), Tannic acid (TA) and Tannic acid loaded microsphere (MSP)

In the X-ray diffractogram of TA powder, sharp peaks at a diffraction angle of $2\theta = 11.9^\circ$, $18.7^\circ$, $20.32^\circ$ and $25.4^\circ$ are present which suggest that the drug is in amorphous state. There were also two strong peaks
in the diffractogram of plain PCL microspheres and TA loaded PCL microspheres at a diffraction angle of $2\theta = 21.26^\circ$, $23.52^\circ$ exhibiting the semi crystallinity nature of microspheres, but the decrease in intensity of the peaks for TA loaded microspheres indicated the change in semi crystallinity of the PCL. The presence of TA intensity peak at $11.92^\circ$ in TA loaded microspheres showing its maintenance of amorphous nature and its stability is unaffected even in the molecular level when encapsulated in microspheres.

### 4.4.11 In vitro Release

Based on the particle size analysis and entrapment efficiency formulations, F3 was selected for in vitro release studies. The particle size of these formulations was in much lower than the range of 1 - 250 $\mu$m, which is ideal as an injectable.

The % cumulative release of tannic acid from PCL microspheres (50mg), in phosphate buffered saline (PBS) solution of pH 7.4 at 37ºC, are shown in Figure 4.16. The formulation F3 showed an initial burst release of 22–32% (of total drug) in the first 7 h followed by a slow and steady release of TA. Initial burst release of TA from microparticles may be attributed to two reasons. First, burst release occurs mainly due to the heterogeneous TA distribution: TA that is either loosely associated with the surface or embedded in the surface layer is responsible for the burst release. Second, morphology of the microparticles causes initial burst: The drugs escape from the polymeric matrices through the pores and cracks that form during the microsphere preparation. Surface-associated drugs are widely known to be the main cause for the initial burst, but direct evidence is seldom found in the literature. The cumulative amount of drug released on day 16 was observed to be 1.4 mg. The time required to release 50% of the encapsulated drug ($t_{50\%}$) from F3 was found to be 212 h (i.e. ~ 8 days). This showed that the drug
could be released in sustained manner for a prolonged period. These results suggest that optimised TA loaded PCL microspheres may be the viable strategy for controlled release of TA in the joint cavity for a month by single intra-articular injection to manage rheumatoid arthritis especially in knee joints.

Figure 4.16  *In vitro* release of tannic acid from PCL microspheres

4.4.12 Collagen Coating of Microspheres

Collagen is well established material that elicits negligible foreign body response when used in implant application. The collagen immobilization on polymeric scaffold surfaces through various surface modification techniques is the current scenario to improve bio-integration of the polymers with the *in vivo* system. Nevertheless, it requires other chemicals or processing methods to modify the surface of polymers to immobilize the collagen covalently. As a model drug system, we have established the collagen coating on to the quercetin loaded PCL microspheres as a strategy to avoid foreign body response of the microsphere in implant applications (Natarajan et al 2012). Using the same strategy, collagen coating on the
surface of TA loaded PCL microspheres by simple adsorption technique was carried out to avoid inflammatory responses at the site of injection. The collagen coating was confirmed through SEM analysis of coated microspheres as shown in Figure 4.17. Here, protein adsorption principle is used for the coating of collagen onto the surface of solid microspheres and characterized. Coating was confirmed through SEM analysis and from the micrograph shown in Figure 4.17, collagen coating was clearly observed.

![SEM micrographs of collagen coated microspheres](image)

**Figure 4.17**   SEM micrographs of collagen coated microspheres

Preparation of tannic acid loaded polycaprolactone microspheres and the prolonged release of tannic acid have been established. The microparticulate system can be injected intra-articularly for the sustained availability of tannic acid to crosslink type II collagen and stabilize articular cartilage.