University of Alberta

Developing a nanoscopic delivery system for interleukin-2

by

Samuel Philip Abraham

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ABSTRACT

The long term objective of this research is to develop a novel formulation of interleukin-2 (IL-2) that improves its therapeutic index. In this study, physical entrapment of IL-2 in core-shell type nanoparticles of methoxy poly(ethylene oxide)–*block*-poly (D, L-lactide) (MePEO-*b*-PDLLA) and covalent linkage of IL-2 to human serum albumin (HSA) nanoparticles were assessed. Size exclusion chromatography showed that there was no significant encapsulation of IL-2 within the core-shell type nanoparticles. For HSA nanoparticles, IL-2 loading efficiency was $\geq$ 90 % and a significant decrease in the number of free thiol groups on nanoparticles (from 6.27 to 3.99 mol thiol per mol HSA) was observed. The HSA conjugated IL-2 was functionally active as evidenced by cytotoxic T lymphocyte line (CTLL-2) bioassay. The results of this study points to a potential for HSA nanoparticles as IL-2 delivery systems.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CFU-GM	Granulocyte-macrophage colony forming unit
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
CTLL-2	Cytotoxic T lymphocyte line
Cys	Cysteine
DLS	Dynamic light scattering
DTNB	5, 5'-Dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
Eo-CSF	Eosinophil colony stimulating factor
FasL	Fas ligand
FDA	Food and drug administration
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPC	Gel permeation chromatography
HIV	Human immunodeficiency virus
^{1}H-NMR	Proton nuclear magnetic resonance
HSA	Human serum albumin
I	Initiator
^{125}I	Radioiodine
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G

IL	Interleukin
Jak	Janus kinase
Kd	Dissociation constant
kDa	kilo Dalton
LAK	Lymphokine activated killer
Mo	Monomer
MAb	Monoclonal antibody
MePEO-*b*-PDLLA	Methoxy polyethylene oxide - *block* - poly (D, L-lactide)
MHC	Major histocompatibility complex
Mn	Number-average molecular weight
Mw	Weight-average molecular weight
MW	Molecular weight
NaI	Sodium iodide
NK	Natural killer
NP	Nanoparticle
PBLA	Poly (β-benzyl-L-aspartate)
PBS	Phosphate buffered saline
PCL	Poly (ε-caprolactone)
PDLLA	Poly (DL-lactic acid)
PEG	Polyethylene glycol
PEG-IL-2	Polyethylene glycol modified IL-2
PEO	Poly (ethylene oxide)
PLA	Poly lactic acid

PLGA	Poly (DL-lactide-co-glycolic acid)
PLLA	Poly (L-lactic acid)
PVA	Polyvinyl alcohol
RCC	Renal cell carcinoma
RES	Reticuloendothelial system
RPMI	Roswell Park Memorial Institute
SCID	Severe combined immunodeficiency
SEC	Size exclusion chromatography
SIL-2	Succinyl IL-2
SSL-IL-2	IL-2 in sterically stabilized liposome
STAT	Signal transducer and activator of transcription
TCA	Trichloroacetic acid
TCR	T-cell receptor
TEM	Transmission electron microscopy
THF	Tetrahydrofuran
TNB	2-nitro-5-thiobenzoic acid
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
V_d	Volume of distribution

Chapter 1

INTRODUCTION

1.1 General features of cytokines

Cytokines are proteins or glycoproteins secreted by a variety of cells. They may exert autocrine or paracrine action i.e, bind to receptors on the membrane of the same cell that has secreted them or attach to receptors on a target cell in proximity to the cytokine secreting cells. After binding to receptors, they initiate signal transduction to the interior of the cell. Thus, cytokines act as intercellular messengers [1].

Initially, cytokines were named based on their cellular source and function. Some cytokines were referred to as interleukins to indicate that they mediate communication among leukocytes. Cohen introduced the term *cytokine* as a general name for substances that activate the immune system [2]. The numerous source and effects of each cytokine has frustrated the attempts to develop a systematic nomenclature.

Cytokines are multifunctional with frequent overlapping functions. The message that a cytokine communicates to a target cell may vary depending on the cytokine, the target cell and other cytokines present. They may communicate to target cells to mature, to proliferate or differentiate, to undergo cell death or to secrete antibodies or other cytokines. Cytokines may have different effects on different target cell, i.e, be pleiotropic. When more than one cytokines mediate the same function, they are said to be redundant. Cytokines can also be synergistic or antagonistic to each other [1, 3, 4].

1.2 Interleukin-2

Interleukin-2 (IL-2) is a pleiotropic cytokine that controls the proliferation and differentiation of the cells of the immune system. It was initially termed T cell growth factor [5]. It is synthesized and secreted primarily by T helper lymphocytes. This glycoprotein allows the *in vitro* expansion of antigen or mitogen activated T cells. *In vivo* it functions as a signal in lymphocyte mitogenesis and has importance in immune responses to tumor antigens. At this level IL-2 acts as an autocrine factor driving the expansion of antigen-specific cells. It also acts as a paracrine factor influencing the activity of other cells like B cells [6] and natural killer (NK) cells [7]. IL-2 promotes B and T cell proliferation and differentiation, and stimulates a cytokine cascade that includes various interleukins, interferons and tumor necrosis factors. For all these activities, IL-2 has attracted extensive attention and in the last two decades, scientists have been exploring its therapeutic potential.

Studies which point out the role of IL-2 in the treatment of solid tumors have generated an interest in its potential clinical use [8, 9]. Low levels of production of IL-2 by T cells from patients with acquired immune deficiency syndrome has also been reported [10]. Partial restoration of T cell function in these patients was obtained by exogeneously given IL-2 [11]. All these studies suggest that IL-2 has a promising role in immunotherapy.

1.2.1 Protein characteristics

IL-2 is a protein of 133 amino acids with a molecular weight (MW) of

15,000-18,000 Da. It is clinically approved for the treatment of renal cell carcinoma (RCC) and metastatic melanoma. It was first characterized biochemically from Jurkat cells, a human T cell leukemic line. Murine and human IL-2 display a homology of 63 percent [12]. IL-2 is synthesized as a precursor protein of 153 amino acids with the first 20 amino terminal amino acids functioning as a hydrophobic signal sequence. The signal sequence is cleaved to produce the mature protein [13].

The amino acid sequence of IL-2 is given in figure 1.1. The protein contains three cysteine residues located at amino acid positions 58, 105 and 125. Two cysteines (Cys), Cys-58 and Cys-105 are involved in intramolecular disulfide bridging to form a biologically active IL-2 molecule [14]. The formation of disulfide bond is essential for biological activity [15-17]. Natural IL-2 is O-glycosylatyed at threonine at position 3 [18]. Varying degrees of glycosylation account for the observed range of MW. However, glycosylation is not essential for biological activity [19]. It promotes elimination of the protein by hepatocytes. Therefore, the removal of the entire carbohydrate component may improve the *in vivo* half-life of the molecule, as has been demonstrated for interferon [20].

The structure of IL-2 is important in order to understand its activity. Studies by Schrader *et al* have shown that IL-2 and granulocyte/macrophage colony stimulating factor (GM-CSF) share significant degree of sequence identity [21]. However, x-ray-derived structure appears topologically different. Hence a three dimensional structure was elucidated for IL-2 which consists of five α helices and two short β strands [22]. The helices are designated A, B-B', C and D

in figure 1.2. There is a short helical segment designated as α. The cysteine 58/105 disulfide link is between helix B and C-D loop. Mutation of cysteine 125 to serine can cause an increase in activity. This may be due to increased stability of the helix by eliminating a sulfur atom or because cysteine 125 is chemically reactive [23].

1.2.2 Biological activity

The biological activities of IL-2 are mediated by the binding of IL-2 to its receptor (IL-2R). The IL-2R is multimeric, consisting of IL-2-specific IL-2Rα (CD25) and IL-2Rβ (CD122), and the "common IL-2Rγ" (CD132) chain [24-26]. The IL-2Rγ is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Only IL-2 and IL-15 have been found to date to utilize the IL-2/15Rβ [27, 28].

IL-2Rα is a 55 kilo Dalton (kDa) glycoprotein having 251 amino acids. It has a short 13 amino acid cytoplasmic tail. This subunit has low affinity for IL-2 (Kd = 10 nM) and it forms the low affinity receptor. This receptor subunit has no signal transducing ability [29]. Cells, which express alpha chains, include activated CD4+ and CD8+ T cells [30, 31], activated B cells [32], glioblastoma cells [33], activated monocytes [34], kupffer cells, macrophages and langerhan cells [35, 36] and tumor cells [37].

IL-2Rβ is a 70-kDa glycoprotein having 525 amino acids. It has a large 286 amino acid cytoplasmic region. This receptor subunit binds IL-2 (Kd = 100 nM) and has signal transducing ability. Cells that express beta chain include activated CD56+ (NK) cells, CD8+ and CD4+ T cells, activated B cells and neutrophils [38].

IL-2Rγ is a 64-kDa glycoprotein having 347 amino acids. It has an 86 amino acid cytoplasmic region. IL-2Rβ and IL-2Rγ interact through their cytoplasmic domains to transduce an IL-2 signal [29]. Cells known to express gamma subunit include monocytes [39], neutrophils [40], CD4+ and CD8+ T cells, NK cells and B cells [41].

The existence of three distinct subunits allows for multiple subunit combinations. The alpha-beta-gamma subunit combination forms the high affinity receptor (Kd = 10^{-11} M). Beta-gamma subunit combination forms the intermediate affinity receptor (Kd = 10^{-9} M). The isolated alpha subunit forms the low affinity receptor (Kd = 10^{-8} M). The intermediate and high affinity forms of the IL-2 receptor can internalize IL-2 and mediate its biologic activity [42-44]. The IL-2Rα and IL-2Rβ have shown to interact with different regions of the IL-2 molecule. The binding interface between IL-2 and IL-2Rα involves amino acids 33-56 and that with IL-2Rβ involves amino acids 11-20 [26]. The interaction surface of IL-2 with IL-2Rγ is composed of small contact patches. The first one involves IL-2 residues serine 127 and 130 and the second is around residue glutamine 126 [45].

The interaction of IL-2 with its receptor leads to a series of signaling events. These include activation of the Janus kinase (Jak) as well as signal transducer and activator of transcription (STAT) pathways [46]. IL-2 induces dimerization of the beta and gamma chains, leading to phosphorylation and activation of Jak-1 and Jak-3. Stat proteins like Stat 1α, Stat 1β, Stat 3 and Stat 5 are thereby phosphorylated and translocate to the nucleus where they bind to

specific DNA sequences, which leads to transcriptional activation of genes [47]. The knowledge of the signalling complexes gives understanding on T cell and NK cell activation and such processes as immunodeficiency states and lymphoid malignancies. It has emerged that mutations in the genes encoding both IL-2Rγ and Jak-3 result in severe combined immunodeficiency (SCID) [48-50]. Therefore it is conceivable that immunosuppression might result if IL-2Rγ/Jak-3 interactions or Jak-3 itself is targeted. This gives a new direction that can be explored for therapy. Such a drug might be useful in the treatment of transplant rejection or autoimmunity.

1.2.3 Mechanism of IL-2 activation

IL-2 displays anti-tumor activity by inducing the proliferation of antigen activated T cells. The activation of a T cell occurs when it recognizes a foreign peptide bound to a major histocompatibility complex (MHC) molecule on the surface of an appropriate target cell. For helper T cell, this appropriate target is an antigen-presenting cell [51]. Also, to activate a helper T cell, the antigen-presenting cell must provide two signals. Signal 1 is provided by foreign peptide bound to class II MHC molecule on the surface of antigen presenting cell. Signal 2 is provided by a co-stimulatory molecule such as B7 [52]. The activated T cells secrete IL-2 and synthesize receptors for IL-2. The secreted IL-2 serves as signal 3. It binds to the receptors, which stimulates the T cells to proliferate as shown in figure 1.3. Moreover, IL-2 causes the activation of NK cells. NK cells differ in several ways from T cells. They are fewer in number than T cells and they do not

express antigen-specific receptors. However, they constitutively express IL-2R. About 90 % of NK cells express IL-2 receptor that has a lower affinity for IL-2, as most of them lack α chain of the IL-2 receptor [31]. NK cells activated by IL-2 can kill cancer cells by antibody-dependent cell-mediated cytotoxicity. They combine through their Fc receptors with the Fc region of immunoglobulin G (IgG) antibodies. The antibodies bind by their Fab region to cancer cell surface antigen [53]. Perforins and granzymes released by NK cells cause the lysis of the target cell. Furthermore, IL-2 can induce the production of secondary cytokines like tumor necrosis factor-α (TNF-α), TNF-β and interferon-γ (IFN-γ) [54]. TNF-α and TNF-β has cytotoxic effects. IFN-γ increases the activity of cytotoxic T lymphocytes (CTL) and NK cells [1].

1.2.3.1 In vitro activity of IL-2

(a) T cells - The T cell growth activity of IL-2 has been used to maintain the long-term growth of T cells *in vitro*. The generally used assay for estimating IL-2 activity measure its ability to cause dose depended proliferation of T cell lines [55]. IL-2 causes the proliferation of mitogen activated primary T lymphoblasts, cytotoxic T cells, helper T cells [56], and T regulatory cells [57]. It has shown to facilitate the generation of both murine and human specific CTL responses [58]. In addition, it can stimulate the production of IFN-γ, TNF and more IL-2 [59-61].

(b) B cells - Activated B cells can express receptors to IL-2. IL-4 or IL-5 can induce IL-2R expression by B cells with resultant proliferation [62]. It can also increase the IgG-producing ability of antigen-specific B cells *in vitro* [63]. With

IL-2 stimulation, antigen-activated B lymphocytes progress through cell cycle and differentiate into antibody-secreting cells [64]. IL-2 may also regulate immunoglobulin E (IgE)-committed B cells, as they are able to respond to IL-2 [65].

(c) Monocytes-macrophages - IL-2 augments the cytotoxicity of human monocytes [66]. Monocytes activated by lipopolysaccharide or interferons express surface IL-2 receptors [67]. Macrophages can be stimulated by IL-2 to secrete TNF *in vitro* and *in vivo* [68]. Moreover, IL-2Rα has been found on cells of monocytes-macrophage series, including cultured monocytes, Langerhan's cells of the skin, Kupffer cells of the liver, cultured lung macrophages and on intestinal lamina proprial macrophages in inflammatory bowel disease [36, 69].

(d) NK and LAK cells - IL-2 augments the proliferative and cytolytic activity of murine and human NK cells. It activates NK and Lymphokine activated killer (LAK) cells to secrete cytokines like IL-1, IFN-γ, and TNF [43, 70, 71]. Thus IL-2 is involved in both innate host responses mediated by NK cells and macrophages and antigen-specific immunity mediated by T cells. In animal models, adoptively transferred LAK cells have shown to mediate regression of hepatic and pulmonary metastasis when IL-2 is administered concomitantly [72, 73]. Although IL-2 can induce LAK cytotoxic activity, the intensity of the response can be regulated by other cytokines including TNF. The failure of low dose IL-2 to induce LAK activity has been shown to be related to their inability to induce TNF production [74].

(e) Other cells - IL-2 has been found to have effects on other cell types.

The presence of IL-2 binding sites and IL-2-like immunoreactive material in the adult rat brain has been described [75]. IL-2 causes the *in vitro* proliferation and differentiation of rat oligodendrocytes and brain glial cells [76]. It also activates osteoclasts *in vitro* to increase acid production and osteoclastic activity [77]. Human mast cells and basophils also express IL-2 receptors [78].

1.2.3.2 In vivo activity of IL-2

(a) Cellular effects - The *in vivo* effects of human recombinant IL-2 have been assessed in animals and humans to evaluate its role in immunotherapy. There does not seem to be much species specificity to its bioactivity. Recombinant IL-2 administered to mice causes the proliferation of T lymphocytes and enhances the activity of endogenous NK or LAK cells [79, 80]. The i.v. injection of LAK cells with recombinant IL-2 in mice with established B16 melanoma led to a marked decrease in lung metastasis and thereby improved survival [81].

The pharmacological effects of IL-2 in humans are numerous. One frequently noticed cellular effect of IL-2 in cancer patients is eosinophilia. This may be due to the eosinophil colony stimulating factor (Eo-CSF) activities of various components, including IL-5 and GM-CSF, and chemotactic factors for eosinophils induced by IL-2 [82]. IL-2 also causes lymphopenia that occurs within 1-2 days after initiation of therapy [83], post-treatment increase in lymphocytes with an increase in NK activity and induction of LAK activity [84, 85].

The study of effects of *in vivo* administration of IL-2 on hematopoiesis showed that the numbers of circulating erythroid, myeloid and multipotential

progenitor cells strongly increased, reaching a maximum after 5 days [86]. Multiple weekly cycles of recombinant interleukin-2 treatment increased peripheral blood granulocyte-macrophage colony forming units (CFU-GM), ranging from 14 to 57 times the baseline values [87].

IL-2 increased the percentage of lymphocytes with the NK phenotype. However, within 10-15 min after the start of an IL-2 infusion, a complete disappearance of all NK lymphocyte subpopulations (including both CD3- CD56+ and CD3+ CD56+ cells with either alpha/beta or gamma/delta T-cell receptor) was observed from peripheral blood of patients. In contrast, the number of T lymphocytes without NK activity (CD3+ CD56-) remained the same. This may be due to the increased adherence of NK and NK-like lymphocytes to the activated endothelium, induced by IL-2 alone or in combination with IL-2 induced TNF-α [88].

Sondel *et al* reported that consecutive weekly cycles of human recombinant IL-2 by intravenous infusion to patients with cancer have shown increase in lymphocyte number and augmented their *in vitro* antitumor reactivity [89]. However, others have shown that not all cancer-bearing hosts respond to IL-2 and LAK cell immunotherapy. It is suggested that IL-2-activated cytotoxic T cells compete with LAK cells for IL-2 and thereby suppress LAK cell responses [90]. Caligiuri et al investigated the consequences of continuous infusions of recombinant interleukin-2 to patients at concentrations that saturated only high affinity IL-2R. They have reported that low dosage regimens can selectively expand human NK cells with only minimal toxicity [84].

(b) Cytokine effects - IL-2 is capable of inducing various secondary cytokines *in vivo*. Significant augmentation of the levels of IL-6 and IFN-γ was seen after IL-2 therapy [91]. The levels of IFN-γ did not correlate with the dose of IL-2. In some other studies IFN-γ has been undetectable [92]. Also, the induction of TNF-α and TNF-β has been observed by some investigators but not others [91, 93]. The greatest increase in circulating cytokines has been found in patients receiving IL-2/LAK therapy [94]. Daily intrapleural injection of IL-2 has shown to increase the levels of IL-6 and IFN-γ within the compartment [91]. Injection into the cerebrospinal fluid of patients also showed similar response [95].

(c) Other effects - Administration of recombinant IL-2 may cause antigenic effects like induction of antibodies specific to IL-2. This may affect the efficacy of repeated therapy. However, studies have shown that development of antibodies did not affect the clinical responses in most patients. Although antibodies were detected in serum samples of almost 50 % of 217 patients in clinical trials, antibodies with neutralizing activity were found only in 7 % of patients. Also, the generation of antibodies did not affect the duration of clinical responses in these patients [96].

IL-2 may cause adverse effects on the humoral immune system. Gottlieb *et al* found that patients receiving IL-2 infusion did not show primary antibody responses to antigen challenge and also reduced secondary response. There was lack of response up to 7 weeks after therapy suggesting that this was not due to capillary leakage, which occurs during treatment by IL-2 [97]. Exacerbation of autoimmunity by IL-2 has been reported in one patient, where autoantibodies

against erythrocytes were found [98]. Reports on the effects of IL-2 in patients are varied and sometimes conflicting.

1.2.4 Pharmacokinetics of IL-2

After intravenous injection, IL-2 preferentially accumulated in liver and spleen. Uptake by liver was rapid and a peak concentration was obtained within 5 minutes [99]. One quantitative estimate of drug distribution is its volume of distribution (V_d). This is a useful parameter in clinical practice because it provides an overall numerical estimate of the extent of drug distribution. After bolus or 2 h i.v infusion of IL-2, the V_d in patients ranged between 6.3 and 7.9 L. This was approximately equal to the total calculated extravascular space [100, 101]. Administration of repeated high doses of IL-2 appeared to increase the V_d. This may be attributed to an increase in the number of IL-2 target cells during the treatment [102].

IL-2 is eliminated by the kidney. Renal catabolism occurs in the following steps: filtration by the glomerular sieve, reabsorption by proximal tubules accompanied by peritubular uptake, degradation in tubular cells and elimination of proteolytic fragments [103]. Cathepsin D, a renal acid protease, is responsible for the degradation of IL-2. Pepstatin A, an acid protease inhibitor, inhibits the degradation of IL-2. As a result of *in vivo* pepstatin treatment, renal cathepsin D activity was greatly inhibited, which in turn reduced the degradation of circulating IL-2, and prolonged serum half-life [104].

After intravenous bolus administration, the level of IL-2 initially

decreased with a half-life of 13 min, followed by a slower phase with a half-life of 85 min [101]. A 1 h infusion also showed similar characteristics [105]. In comparison of administration of IL-2 by bolus versus continuous intravenous infusion, the National Cancer Institute Biological Response Modifiers Program reported that continuous intravenous infusion resulted in higher lymphocytosis and number of LAK cells [106]. Intravenous administration of 16.7 $\mu g/m^2$ of body surface area of recombinant IL-2 manufactured by Amgen Corporation resulted in plasma concentration that decayed for approximately 45 minutes. IL-2 administered subcutaneously in the same dose has subsequent decay at a $t_{1/2}$ of 2.8 hs. Thus, subcutaneous administration is preferable to intravenous administration, because it allows plasma IL-2 concentration sufficient to bind more than half of receptors expressed by antigen activated T cells [107].

1.2.5 *Toxicity of IL-2*

The clinical use of IL-2 is limited by systemic side effects that may lead to organ dysfunction and failure. The toxicities of IL-2 therapy are dose and schedule related. Specific adverse effects of IL-2 include systemic, dermatologic, cardiopulmonary, renal, gastrointestinal, neuropsychiatric, hematological and endocrinological toxicities.

Systemic effects include fever and chills. IL-2 was unable to induce prostaglandin E2 synthesis in hypothalamic cells or fibroblasts *in vitro*. This indicates that IL-2 is not pyrogenic by itself. It causes fever by inducing release of pyrogenic cytokines, in particular TNF-α [108]. Dermatologic effects manifest as

macular erythema. This occasionally became generalized (i.e., erythroderma) [109]. Cardiac toxicities include arrhythmias, ischemia, myocarditis and hypocontractility. Supraventricular tachycardia and atrial fibrillation was reported in 10 % or more of patients [110]. Renal effects include oliguria accompanied by low sodium excretion and high plasma rennin activity. Generally, renal dysfunction reversed after the therapy was discontinued [111]. Gastrointestinal effects include nausea, vomiting, and anorexia. Intrahepatic cholestasis with elevations of bilirubin to 2-7 mg/dL range was observed in most patients [112]. Neurologic toxicity includes hallucinations and seizures. Patients may become agitated, combative and disoriented [113]. These effects can be caused due to cerebral edema. Hematologic effects are very common and include anemia, lymphopenia, eosinophilia, and thrombocytopaenia. The reason for this hematopoietic suppression may be due to the induction of IFN-γ and TNF which suppresses progenitor cells [114]. Endocrinologic effects include increased levels of cortisol, corticotropin, prolactin and growth hormone [115]. The reason for this physiologic response is not known.

One important toxicity of IL-2 administration is capillary leak syndrome. The reason may be that IL-2 itself has a direct influence on blood vessels. Another cause is IL-2 activated lymphoid cells that mediate damage to vascular endothelium. Capillary leak syndrome has also been reported in mice treated with recombinant IL-2 [116].

The mechanism of toxicity induced by IL-2 therapy involves many factors. It is generally due to physiological changes that result from initiation of

inflammatory response mediated by other cytokines [117]. NK cells are stimulated by IL-2 to secrete a series of cytokines including IFN-γ, TNF-α and GM-CSF. These cytokines are activators of macrophage. The activated macrophages secrete IL-1 and IL-6. These secondary cytokines activates vascular endothelium and increases its permeability. As well macrophages activated by cytokines begin to express high levels of nitric oxide synthetase, which causes the production of nitric oxide. Nitric oxide increases vascular permeability [118]. Furthermore, activated neutrophils bind to endothelial cells and they synthesize and release thromboxane inducing vasodilatation. Neutrophils also release lysosomal enzymes and oxygen radicals resulting in endothelial injury [119]. The complement proteins C3a and C5a also mediate vasodilatation and increase vascular permeability [120]. All these factors lead to damage of endothelial cells. This damage causes leakage of plasma proteins and fluid from the vascular compartment into the interstitium. The fall in intravascular volume results in tachycardia, hypotension and accumulation of fluid in the interstitial space which causes other adverse effects. Approaches to reduce toxicity include neutralizing antibodies to secondary cytokines produced, different routes of administration, schedules of administration, doses of drug and delivery systems.

1.2.6 Therapeutic uses of IL-2

An estimated 36,000 new cases of RCC and 59,000 new cases of melanoma will occur in United States in 2005 [121]. Many of these patients may

develop metastatic disease. The median survival time for metastatic RCC patients after diagnosis is 8 to 12 months [122]. Surgery and radiation therapy are mainly for palliation and to improve quality of life. Thus, effective therapies are limited for both diseases.

Interleukin-2 has shown some promising results for the treatment of RCC and melanoma. Accordingly, Food and Drug Administration approved it for use in these malignancies. A long-term survival update of 255 patients with RCC treated with high-dose i.v. infusion recombinant IL-2 showed that the treatment is highly effective. Objective responses were noted in 37 of 255 patients (15 %) with 17 complete responses (7 %) and 20 partial responses (8 %). Median survival time for all 255 patients was 16.3 months. 10 to 20 % of patients remained alive 5 to 10 years after treatment with high-dose IL-2 [123]. Similar response rates have been observed for low-dose IL-2 regimens [124]. Treatment with subcutaneous IL-2 at low doses in combination with IFN-α was found to be as effective as high-dose i.v. bolus IL-2 [125-127]. For RCC patients with pulmonary metastases, who have no other treatment option available, the use of IL-2 by inhalation in combination with low dose subcutaneous IL-2 was efficacious and safe. Inhalation of IL-2 can cause dose-dependent cough due to local toxicity, but hardly any systemic adverse effects were found [128, 129].

For melanoma, a long-term survival update of 270 patients treated with high-dose i.v. bolus recombinant IL-2 reported that tumor responses were seen in 16 % of patients, with complete responses in 17 (6 %) and partial responses in 26 (10 %). Median follow-up time for surviving patients exceeded 7 years [130].

These data indicate that IL-2 therapy produces lasting responses in some patients with metastatic melanoma. IL-2 has also been studied in the treatment of colorectal, ovarian and bladder cancers, non-hodgkin's lymphoma and acute myeloid leukemia [131]. The majority of studies have been done with RCC and melanoma patients where IL-2 has shown a definite role in therapy. Nevertheless, further clinical research is needed to get a clear understanding of its efficacy in other malignancies.

1.2.7 Current understanding of IL-2 function

IL-2 was originally thought to be a straightforward T cell growth factor. Therefore, the IL-2 deficient mice was expected to be profoundly immunodeficient. However, it was a surprise to find out that IL-2 deficient mice developed massive lymphoproliferation and lethal autoimmunity [132-134]. Between 4-6 weeks of age, IL-2 and IL-2Rα-deficient mice showed massive enlargement of peripheral lymphoid organs due to polyclonal expansion of T and B cells. Between 8-20 weeks, many of these mice died from severe anemia. The rest developed an inflammatory bowel disease that is similar to ulcerative colitis in humans [132, 135]. These findings suggested that IL-2 is not only a T cell growth factor, but also essential for regulating self-tolerance.

One mechanism by which IL-2 functions to regulate T cell response is by activation induced cell death. T cell response to antigen involves two phases – an activation phase and a proliferation phase. The activation phase leads to the production of IL-2 and its high affinity receptor [136]. In the proliferation phase

IL-2 binds to its receptor and causes cell cycle progression. Once the T cells are driven to late G1 or S phase of cell cycle, they are susceptible to apoptosis [137, 138]. Studies have shown that both CD4 and CD8 T cells exposed to IL-2 are susceptible to apoptosis after antigen receptor stimulation [139]. Thus IL-2 provides a feedback regulatory mechanism by inducing apoptosis in T cells. This limits T cell expansion when the antigen is repeatedly encountered.

T cell apoptosis takes place by the expression of death cytokines, particularly Fas ligand (FasL) [140, 141]. In resting T cells, the genes for FasL is weakly induced by T-cell receptor (TCR) stimulation, but in IL-2 activated T cells, it is dramatically increased [142]. Cell death is mediated by FasL/Fas interactions. Thus, one mechanism by which IL-2 potentiates Fas-mediated apoptosis is by enhancing the transcription and surface expression of FasL. Furthermore, IL-2 suppresses the transcription and synthesis of a protein, designated FLIP, which is an inhibitor of apoptosis [143]. Hence, the ability of IL-2 to raise the expression of a pro-apoptotic molecule, FasL, and to suppress an inhibitor of apoptosis, FLIP, probably explains its function in enhancing T cell apoptosis.

1.2.7.1 IL-2, T regulatory cells and tolerance

In vivo studies of IL-2Rα-deficient T cells have shown reduced expansion in response to antigen. However, effect on T cell apoptosis was intact [144]. Furthermore, mice expressing IL-2Rβ in the thymus but not in the periphery where activation induced cell death occurs did not develop autoimmunity [145]. These observations suggested that activation induced cell death may not be the

main mechanism by which IL-2 regulates self-tolerance.

Recent studies indicate that IL-2 is involved in the development of $CD4^+$ $CD25^+$ regulatory T cells (Tregs). $CD4^+$ $CD25^+$ Tregs develop in the thymus and migrate to the periphery. They constitute 5 – 10 % of peripheral $CD4^+$ and less than 1 % of $CD8^+$ T cells in humans and mice [146-149]. Activated T cells also express CD25, but this is generally of lower magnitude compared with Tregs [150, 151]. Tregs express all of the subunits that are needed for a functional high affinity IL-2 receptor [152].

IL-2 promotes Treg cell growth *in vitro*. Also, it was found that administration of IL-2 or re-introduction of IL-2 producing cells to IL-2 deficient mice restored the production of Treg cells [153-156]. Furthermore, Malek et al have shown that IL-2 signaling is important for the development of Treg cells in the thymus [145, 152]. IL-2Rβ expression in thymus but not in the periphery of IL-2Rβ deficient mice restored development of $CD4^+$ $CD25^+$ Tregs and prevented lethal autoimmunity. Suppression of autoimmunity was also observed after adoptive transfer of wild-type Treg cells into IL-2Rβ deficient mice. Also, there was clonal expansion of these donor cells. This was due to the expression of a functional IL-2R by donor Treg cells and IL-2 production by the host [145, 152]. Thus, IL-2-IL-2R signaling appears to be required for the production and proliferation of Treg cells. These Tregs in turn generate self-tolerance. This strongly favors the notion that the main mechanism by which IL-2 regulates tolerance is by promoting the development of T regulatory cells.

In vivo, IL-2 contributes to both tolerance and immunity. Tolerance is promoted by activation induced cell death and development as well as proliferation of Treg cells, as described above. With respect to immunity, IL-2 is more important at a later stage of the effector response. Studies have shown that the delivery of IL-2 during a viral infection was most beneficial during the contraction phase of the immune response [157]. Here, it increased the proliferation and survival of long-lived virus-specific T cells. However, IL-2 given during the expansion phase resulted in reduction of antigen-specific $CD8^+$ T cells. Hence, excess IL-2 during the early phase of viral infection may induce rapidly dividing effector $CD8^+$ T cells to undergo apoptosis. Therefore, to enhance T cell responses *in vivo*, timing of IL-2 administration and proliferative status of T cells are vital parameters [157].

The new understanding of IL-2 function prompts one to re-assess its use in clinical settings. Administration of IL-2 may not always result in enhanced immunity. IL-2 may also increase the number or effectiveness of Treg cells. This can limit immune responses, which is undesirable in cases where augmentation of immunity is the aim of treatment. In this context, depletion of $CD25^+$ Tregs using CD25-specific antibodies might be beneficial. Removal of immunoregulatory $CD4^+$ $CD25^+$ T cells has shown to elicit tumor immunity in mice [158]. In contrast, the ability of IL-2 to promote the development of Tregs may be used for the treatment of autoimmune diseases. The induced or expanded population of Tregs can inhibit harmful responses against self-antigens. Thus, IL-2 therapy has the potential to manipulate T cell responses for evoking immunity or self-tolerance.

1.3 Formulations of Interleukin-2

High systemic doses of IL-2 are usually given in order to achieve anticancer activity. But the high doses cause severe toxicity as described above and the therapeutic results are limited. Hence different strategies have been explored to formulate and deliver IL-2 to reduce its serious side effects. To increase therapeutic efficacy, increase half-life and to decrease the amount of IL-2 exposed to healthy tissues, most formulations are designed to achieve a localized or sustained mode of delivery. The major formulation approaches used to date for IL-2 delivery include microspheres, liposomes, block copolymers and PEGylated IL-2.

Researchers have evaluated the use of biodegradable poly (DL-lactide-co-glycolic acid) (PLGA) microspheres for the sustained release of interleukin-2, succinyl IL-2 (SIL-2) and polyethylene glycol modified IL-2 (PEG-IL-2) [159]. An injectable delivery system that could continuously deliver 2-3 % PEG-IL-2 per day for over 20 days was developed. The incorporation of human serum albumin (HSA) as an excipient in the formulation prevents aggregation and enhances the release of IL-2 from the delivery system.

In another study, biodegradable poly lactic acid (PLA) microspheres were used to formulate IL-2 [160]. It is believed that these microspheres degrade homogeneously by bulk erosion. The *in vitro* release of IL-2 from the microspheres showed an initial rapid release for 3 days followed by an exponential decline in the release. The therapeutic effect of IL-2 loaded particles was evaluated by co-injecting the microspheres and the tumor cells

subcutaneously into SCID mouse model. Injection of 50 μg microspheres loaded with IL-2 mixed with 2×10^6 tumor cells resulted in the complete suppression of tumor growth in eight of ten mice. In control untreated mice, tumors grew in nine of ten mice. In contrast, bolus injections of poly(ethylene glycol)/IL-2 was not successful in suppressing tumor growth [160]. The antitumor effect of the released IL-2 was mediated by the mouse NK cells. Thus, PLA microspheres can be used to deliver IL-2 in a sustained release manner. One problem with this formulation is the denaturation of the protein during the encapsulation process, which can lead to a decrease in the bioactivity of IL-2.

In a separate study, an injectable polymeric system for the long-term localized (intratumoral) delivery of IL-2 was developed. IL-2 was encapsulated into polymeric matrices formed by the coacervation of gelatin and chondroitin-6-sulfate [161]. Systems made using gelatin and chondroitin-6-sulfate are biocompatible. They can be made to encapsulate IL-2 under conditions that will retain its activity. Furthermore, collagenase secreted by tumor cause an increased rate of polymer degradation. This exposes the tumors to high IL-2 concentration [161]. IL-2 delivery from these microspheres protected mice from primary gliosarcoma and murine metastatic melanoma, brain and metastatic liver carcinoma. The advantage with this formulation is that protein denaturation does not occur during encapsulation process. Moreover, the majority of IL-2 delivered remains in the organ, in which they are injected, thereby reducing systemic toxicity.

IL-2 delivery by liposomes have also been studied. Researchers have

demonstrated that compared to free IL-2, liposomal IL-2 can give increased therapeutic efficacy against murine tumors [162, 163]. Compared to free IL-2, dimyristoyl-phosphatidylcholine liposomes containing recombinant IL-2 have shown increased survival and reduced numbers of pulmonary metastases in mice bearing metastatic MCA-106 sarcoma by intrathoracic route. The enhanced activity of IL-2 via liposome delivery may be due to a sustained mode of IL-2 release by liposomal formulation, increased half-life of IL-2 by liposomes or better activation of macrophages by liposomal delivery. No signs of severe systemic toxicity of IL-2 were seen [163].

Furthermore, studies by Vaage and Mayhew demonstrated that repeated peritumor administration of IL-2 liposome-gel preparation results in both local and systemic therapeutic effects in MC2 mouse mammary carcinoma [162]. The liposome preparation was effective due to the sustained release of IL-2 from this formulation.

Studies have been done to investigate the mode of IL-2 association with liposomes. This is an important aspect to obtain stable preparations with active IL-2 for *in vivo* use. It was hypothesized that pH values below the isoelectric point of IL-2 and electrostatic attraction between IL-2 and negatively charged bilayers is enough to get high incorporation efficiencies of IL-2 in liposomes [164]. In addition, stable retention of encapsulated protein, clearance from the circulation and access to target tissues have been shown to be closely related to the size and composition of liposomes [165-167]. Liposomes larger than 50 to 200 nm in diameter are too large to pass through the walls of normal blood vessels, but they

can pass gaps in newly formed vessels, such as those in tumors. Liposomes ranging in size from 50 to 200 nm therefore have the potential to deliver the encapsulated drug more selectively to tumors, taking advantage of leaky tumor capillaries [168, 169]. Rapid disintegration of liposomes *in vivo* can be overcome by the construction of stabilized liposomes [170] in which the liposomal surface is coated with an inert polymer such as polyethylene glycol (PEG) covalently linked to the phospholipids. This makes the surface of liposomes hydrophilic, reducing opsonization by plasma proteins and prolongs liposome circulation time. Kedar *et al* demonstrated that IL-2 could be successfully encapsulated in long-circulating sterically stabilized liposomes (SSL-IL-2) [171]. SSL-IL-2 was more effective than free IL-2 in increasing leukocyte number in the blood and spleen. Furthermore, in mice with advanced metastatic carcinoma previously treated with chemotherapy (cyclophosphamide), the survival was two to six times greater following administration of SSL-IL-2 as compared with free IL-2 [171]. Recently, Kanaoka *et al* have described a novel and simple type of liposome carrier for IL-2 [172] with a strong association between small (30 nm) hydrophobic liposomes and IL-2. IL-2 remained biologically active after mixing with liposomes. After intravenous administration to mice, liposomal IL-2 was eliminated half as slowly as free IL-2 from the systemic circulation. Liposomes delivered 13 and 18 times more IL-2 to the liver and spleen, respectively. After subcutaneous administration of liposomal IL-2 to mice, the mean residence time of IL-2 in the systemic circulation was 8 times more than this parameter for free IL-2. Liposomal IL-2 is easy to prepare and pharmaceutically convenient. Besides, liposomal IL-2 may be

combined with other drugs to provide better therapeutic response to IL-2 therapy.

Block copolymers have been studied for the sustained delivery of IL-2. The commercially available block copolymer, poloxamer 407, was evaluated for this purpose. Poloxamer 407 was selected as the parenteral vehicle because of its reverse thermal gelation properties [173]. It forms a semisolid gelatinous matrix following an increase in temperature from 8°C to 22°C. Also, it lacks inherent toxicity to muscle tissue following single and multiple dose injections of the vehicle to rabbits [174]. The bioactivity of recombinant IL-2 was maintained following incubation in poloxamer 407 (33 % w/w) at 4°C and 22°C for 72 h. It is considered that poloxamer 407 stabilizes the protein. The *in vitro* release studies showed that the release of IL-2 at 22°C followed zero order kinetics. When given to mice intraperitoneally no toxicity was reported either from recombinant protein or polymer vehicle [173]. When the formulation is injected intramuscularly, a depot is formed at the site of injection, which acts as a reservoir for the incorporated drug. Drug molecules will be released continuously from the reservoir at a constant rate leading to prolonged constant drug levels in blood. In this system, an increase in the concentration of poloxamer increases the elasticity of the gel and decreases sol-gel transition temperature. The strong concentration dependence of the sol-gel phase transition is a limitation in the use of this formulation.

PEG is a hydrophilic, non-toxic polymer that has received Food and Drug Administration (FDA) approval as a vehicle or base for pharmaceutical, food and cosmetic applications. Recombinant IL-2 can be covalently modified by PEG to

give PEG-IL-2. The bioactivity of IL-2 is retained after PEGylation [175]. Pharmacokinetic studies of PEG-IL-2 formulation showed higher plasma levels for PEG-IL-2 in comparison to free IL-2 [176]. Moreover, PEG (4k – 20k) modified IL-2 showed decreased clearance by kidney [177]. Conjugation increases the size of the protein due to hydration of PEG, decreases glomerular filtration of IL-2 by kidneys and leads to a longer half-life for IL-2 [176]. Another reason for reduced clearance may be due to the fact that PEG protects the protein from hydrolysis. Also attachment of PEG to IL-2 reduces its immunogenicity in rabbits, mice and humans [178]. PEG-IL-2 exhibited prolonged immunostimulatory effects in patients with human immunodeficiency virus (HIV) type 1 infection [179]. A combination regimen of high dose IL-2 followed by low dose PEG-IL-2 was tested and devised for the treatment of patients with RCC and melanoma [180]. One limitation with PEGylation is that cytokines like interleukin-2 have few available sites for attachment to PEG. It is known that coupling of more number of low MW strands of PEG can decrease the receptor binding activity of IL-2 *in vitro* [176]. This can reduce the potency of the cytokine. Also, the formulation does not reduce the toxicity of IL-2.

1.4 Block copolymer core-shell type nanoparticles for drug delivery

Copolymers are polymers formed from different monomer units. The monomer units can be arranged in different ways forming four types of copolymers: random copolymers, alternating copolymers, graft copolymers, and block copolymers. Block copolymers are further classified into three types: the AB

type, the ABA type and the $(AB)_n$ type, based on the alternating arrangement of their components [181]. The AB and ABA type are most often studied for drug delivery.

1.4.1 Characteristics of core-shell type nanoparticles for drug delivery

When amphiphilic AB type block copolymers are placed in an aqueous solvent, they self assemble to form core-shell micelles. The hydrophobic segment of the polymer forms the core and the hydrophilic segment forms the shell. The core usually consists of a polymer such as poly (β-benzyl-L-aspartate) (PBLA), poly (DL-lactic acid) (PDLLA) or poly (ε-caprolactone) (PCL) [182]. The shell is mostly composed of poly (ethylene oxide) (PEO). In most cases, the hydrophobic portion has a MW of less than 2000 g/mol and the hydrophilic PEO portion has a MW of 1000 – 12000 g/mol [183].

The formation of distinguished core-shell architecture by these particles makes them very useful for the delivery of drugs. The hydrophobic core of the particle serves as a reservoir for hydrophobic drugs, which suffer from poor water solubility [183]. The hydrophilic shell facilitates dispersion, prevents interparticle aggregation and reduces protein adsorption and provides stealth properties resulting in prolonged blood circulation for the carrier, It protects the drug loaded inside core against hydrolysis and enzymatic degradation [184, 185]. The particles have a small size (less than 100 nm), which prevents their uptake by reticuloendothelial system (RES) and facilitates their extravasation at leaky capillaries nearby solid tumors [186]. Additionally, the small size of carriers

prevents embolism in the capillaries and makes the carrier safe. The small size of polymeric micelles is advantageous from pharmaceutical perspective, because sterilization of the carrier can easily be achieved through filtration [183].

Without being sufficiently biocompatible, drug delivery systems may not be appropriate for human use. Polyesters like PDLLA have been used for long periods of time and evidence supports that their use in humans is safe. Other polyesters and polyaminoacids can be hydrolyzed or enzymatically degraded into biocompatible monomers [186].

Block copolymer micelles have been used to encapsulate various molecules for drug delivery including adriamycin, amphotericin B, cyclophosphamide, cyclosporin A, indomethacin, paclitaxel, lysozyme and DNA [186-188]. A model protein, HSA, has been shown to be entrapped in biodegradable nanospheres (200 nm) prepared from amphiphilic diblock poly(ethylene oxide)–*block*-poly(lactic acid) [189]. There can be many factors that influence the loading efficiency of drugs in block copolymer nanoparticles. The main factor identified was the compatibility between the drug and the core-forming block [190]. Other factors that may influence drug loading are length of the core and shell forming block and size or molecular volume of the drug [190]. Nagarajan et al. have shown that the amount of solubilizate incorporated in core-shell type particles decreases with an increase in the molecular volume of the solubilizate [191].

The release of drug loaded inside block copolymer micelles is determined by the rate of drug diffusion, the degree of biodegradation of the copolymer and

the stability of the particles. However, if the particles are stable and the biodegradation rate is slow, the rate of drug release will be influenced by the strength of the interaction between the drug and core of the polymer, the physical state of the particle core, the amount of drug loaded, the molecular volume of the drug and the localization of the drug within the particle. As the strength of the interaction between the drug and core of the polymer increases, the release rate will decrease. Also, drugs will move faster out of cores that are liquid-like because these cores have an increased mobility compared to glassy cores [190]. Furthermore, when the amount of drug entrapped increased, the release rate decreased as shown in the case of methoxy poly(ethylene oxide)-*block*-poly(ε caprolactone) block copolymer particles containing indomethacin [192]. This may be due to increased hydrophobic interactions between the drug and core of the polymer. Drugs with higher molecular volumes will show a lower release rate due to low diffusion coefficient [190]. Finally, the location of the drug within the particle, which is largely dependent on the water solubility of the drug, may affect the rate of drug release from the polymeric micelles. While hydrophobic drugs are loaded in the core of the particle, hydrophilic drugs tend to be in the shell or shell-core interface. Due to mobility of the shell, hydrophilic drugs are released rapidly, showing a 'burst release' immediately after contact with the release medium [190].

1.5 Protein based nano and microparticles for drug delivery

Nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm. They consist of macromolecular materials in which the active principle is

dissolved, entrapped or to which the active material is adsorbed or attached. Microparticles are similar particles in the size range of 1 μm to 1000 μm [193]. Protein nanoparticles have attracted considerable attention as potential drug delivery systems. In this context, albumin has been most extensively used.

1.5.1 Albumin as a biopolymer for drug delivery

Albumin is a protein that is found in almost all living body tissues. Commercially it is obtained from egg white, bovine serum and human serum [194]. HSA is the most abundant protein in blood plasma. One of its outstanding properties is the unique ability to bind a variety of ligands. For instance, fatty acids are insoluble in blood, but albumin binds to fatty acids and function as their carrier in blood. Albumin binds metabolites like bilirubin and decreases their toxicity [195]. This property of albumin would have immense therapeutic potential as it can accommodate a wide variety of drugs in a nonspecific fashion.

HSA is a single polypeptide chain of 585 amino acids with a MW of 66,439 Da [196]. It is synthesized by liver and has a half-life of 19 days in circulation [195]. The protein has three domains (I, II and III) as shown in figure 1.4. Its sequence is characterized by a unique arrangement of disulfide double loops that repeat as a series of triplets in the middle and third domains [195]. In the first domain, there are 2 disulfide double loops and 1 single disulfide bridge. Thus, there are 17 disulfides forming the loops. The cysteine residue at position 34 bears the only free thiol group of the protein [197].

The use of albumin nanoparticles in drug delivery was first suggested by

P.A. Kramer [198]. The two main techniques reported for the preparation of particles are emulsification and phase separation based methods [199]. In emulsification method, aqueous solution of albumin is emulsified in oil (e.g., cottonseed oil) and homogenized by sonication. The emulsion is then added dropwise to a large amount of preheated oil (> 120^0 c) while stirring. This causes the evaporation of water contained in the droplets and denaturation of albumin, which coagulates in the form of nanoparticles. Subsequently, the particles are washed using organic solvents (e.g., ether, ethanol) to remove the oil. The disadvantages of this method includes the use of large amounts of organic solvents for purification of particles and the difficulty to obtain small nanoparticles (< 500 nm) with narrow size distribution due to instability of the emulsion. The phase separation method involves desolvation of the protein followed by cross-linking of the particles. In the method proposed by Marty et al [200], nanoparticles can be prepared by slow addition of a desolvating agent (e.g., ethanol) to aqueous solution of albumin. After a certain extent of desolvation is achieved, protein aggregates are formed. The particles are obtained by subsequent cross-linking of the aggregates using reagents such as formaldehyde or glutaraldehyde. The cross-linking process involves the formation of covalent bonds between lysine amino groups of protein and the aldehyde leading to stabilization of the particles [199].

Drugs can be bound to previously prepared empty nanoparticles by covalent coupling or adsorption. They may also be entrapped inside the particles physically [201]. HSA offers various target sites for covalent linkage of drugs like the ε-amino groups of lysine, carboxyl groups of asparaginic and glutaminic acid,

hydroxyl groups of tyrosine and thiol groups of cysteine [202]. In the case of glutaraldehyde cross-linked particles, the remaining carbonyl residues can also be used as potential sites for drug conjugation. Depending on the nature of the covalent bond, different drug release behaviors may be achieved. Mostly, covalent coupling yields a much slower release rate than adsorption [201].

Like other colloidal drug delivery systems, albumin nanoparticles are taken up by RES after intravenous injection. In a study by Scheffel et al [203], the body distribution of ^{99m}Tc-labeled HSA nanoparticles after i.v. injection to mice was evaluated. The percentage of the injected dose found in liver and spleen after 10 min was 86 and 1.8 %, respectively. The adsorption of serum proteins to the particle surface strongly facilitates their uptake by phagocytic cells in RES organs like liver. The coating of particles with poloxamers and poloxamines (e.g., poloxamer 407, poloxamine 908) or PEO-based surfactants such as polysorbates (e.g., polysorbate 80) may reduce their uptake by RES and increase their concentration in other organs and tissues as was observed for polystyrene or poly (methyl methacrylate) nanoparticles [199]. Furthermore, the extent and rate of carrier uptake by RES is influenced by carrier size. The dependence of opsonization extent on carrier size was evaluated by pretreating serum with empty liposomes of various sizes The extent of opsonization and RES uptake was decreased with a decrease in particle size from 800 nm to 200 nm, and phagocytic uptake was less for particles below 200 nm [204, 205].

Albumin-based drug carrier systems have extensively been used as drug delivery systems. Table 1.1 lists various drugs that have been bound to albumin

and albumin nano or microparticles. Targeting of drugs to specific sites *in vivo* using the albumin particles has also been investigated. The incorporation of magnetic Fe_3O_4 particles of 10-20 nm size in diameter into albumin microspheres enabled the preparation of magnetically responsive particles. These particles loaded with doxorubicin and guided externally by a magnet to the target site (rat tail Yoshida tumor) have shown to achieve a significant remission of Yoshida tumors [206]. Coating of albumin nanoparticles with monoclonal antibodies (MAb) is another approach to provide targeting ability to the albumin particles [207]. The MAb decorated particles showed more accumulation in the targeted tumoral tissue of Lewis lung carcinoma-bearing mice.

Over the past years peptide and protein drugs have been gaining more and more importance. In many cases these drugs are quite efficiently bound to nanoparticles. Studies were done to evaluate the capacity of bovine serum albumin (BSA) nanoparticles as carriers of IFN-γ [208]. For this purpose, IFN-γ was adsorbed to the particles. The cytokine was associated with the particles mainly by electrostatic interactions. The adsorbed protein was able to improve the priming effects of IFN-γ on the nitric oxide production by macrophages. In a separate study, apolipoprotein E was coupled to nanoparticles made of HSA to facilitate drug delivery to brain [209]. The protein was chemically bound via linkers to loperamide-loaded HSA nanoparticles. The results indicate that apolipoprotein E attached to the surface of nanoparticles facilitate transport of drugs across the blood-brain barrier possibly after interaction with lipoprotein receptors on the brain capillary endothelial cell membranes. Furthermore, BSA

microspheres were studied for the development of implantable insulin delivery system [210]. A subcutaneous injection of entrapped insulin in the particles produced elevated blood insulin levels in diabetic rats for more than 2 months. The results suggest that a long-acting insulin may be produced by the entrapment of insulin within a biodegradable matrix.

1.6 Thesis proposal

1.6.1 Rationale

Interleukin-2 is a cytokine that plays an important role in immunological events. However, its widespread clinical use has been limited by a narrow therapeutic index. The toxic effects of IL-2 are mainly due to its broad biodistribution arising from high plasma concentrations, whereas its therapeutic effects are dependent on availability at the tumor site. Novel delivery approaches that can limit the systemic concentration of IL-2, while at the same time increase its bioavailabilty at the tumor microenvironment would enhance its therapeutic index. This approach can boost the efficacy of IL-2 and also overcome toxicity associated with its therapy. Thus, the goal of this research was to develop a novel carrier for IL-2 that can limit the systemic concentration of IL-2, but enhance its delivery to tumor site. The potential of polymeric micelles and HSA nanoparticles as tumor targeted delivery system for IL-2 was investigated in this study. We studied the extent of IL-2 encapsulation in polymeric micelles and IL-2 conjugation on HSA nano-particles. The biological activity of the IL-2 conjugated HSA nanoparticles was also investigated.

1.6.2 General objective:

To achieve a novel formulation based on biodegradable nanoparticles for the delivery of IL-2.

1.6.3 Hypothesis:

1) Methoxy poly(ethylene oxide)–*block*-poly(D, L-lactide) (MePEO-*b*-PDLLA) micelles can act as efficient carriers for the physical encapsulation and delivery of IL-2 and

2) IL-2 can efficiently be conjugated to the surface of HSA nanoparticles by disulfide bond formation and retain its bioactivity after conjugation.

1.6.4 Specific aims:

- To prepare IL-2 loaded micelles of MePEO-*b*-PDLLA and assess the characteristics of the IL-2 encapsulated MePEO-*b*-PDLLA micelles.
- .To prepare IL-2 conjugated HSA nanoparticles and assess the characteristics of the IL-2 conjugated HSA nano-particle.
- To assess the *in vitro* bioactivity of IL-2 incorporated nano-carriers making comparison with the bioactivity of soluble IL-2.

Table 1.1 Albumin-based drug carrier systems for drug delivery

Delivery system	Drug	Reference
Albumin conjugates	Mitomycin C	[211]
	Taxol	[212]
	Chlorambucil	[213]
	Methotrexate	[214]
	Doxorubicin	[215]
Drugs bound to nano or microparticles	Doxorubicin	[206]
	5-Fluorouracil	[216]
	Mercaptopurine	[198]
	Methotrexate	[217]
	Metronidazole	[218]
	Primaquine	[218]
	Triamcinolone	[219]

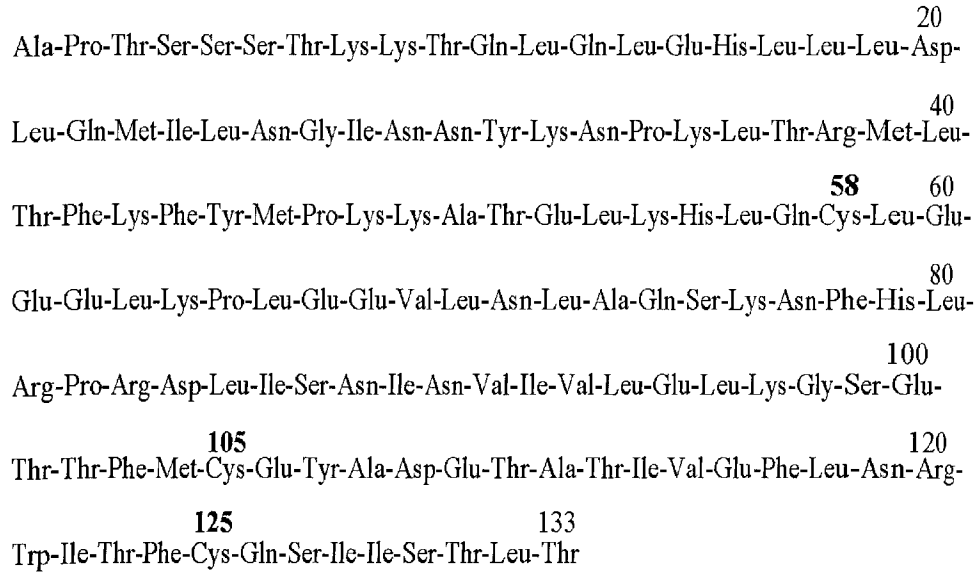


Figure 1.1: Amino acid sequence of IL-2*

*Figure modified from Ref. [220].

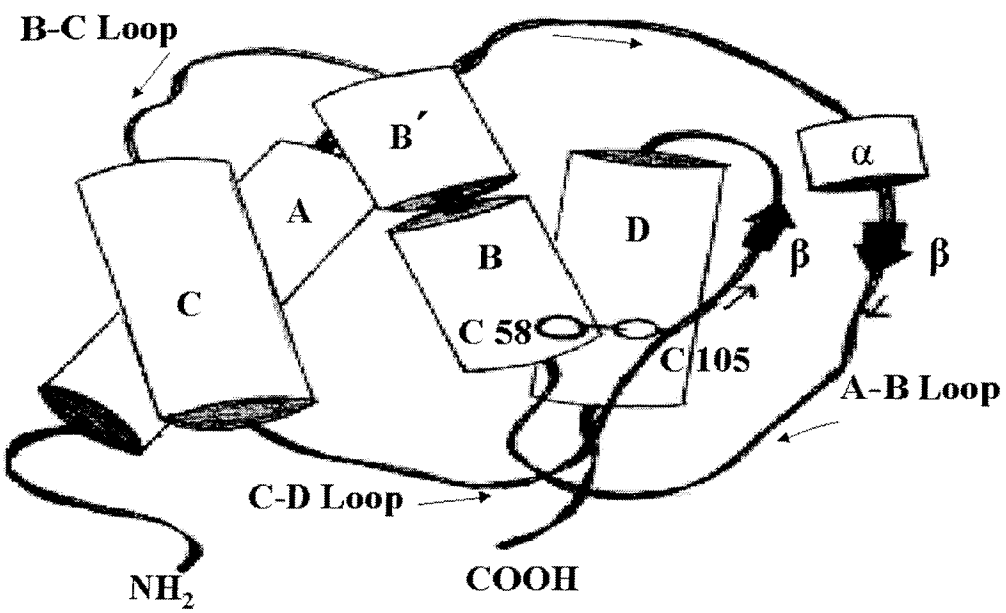


Figure 1.2: Three-dimensional structure of IL-2*

*Figure modified from Ref. [22].

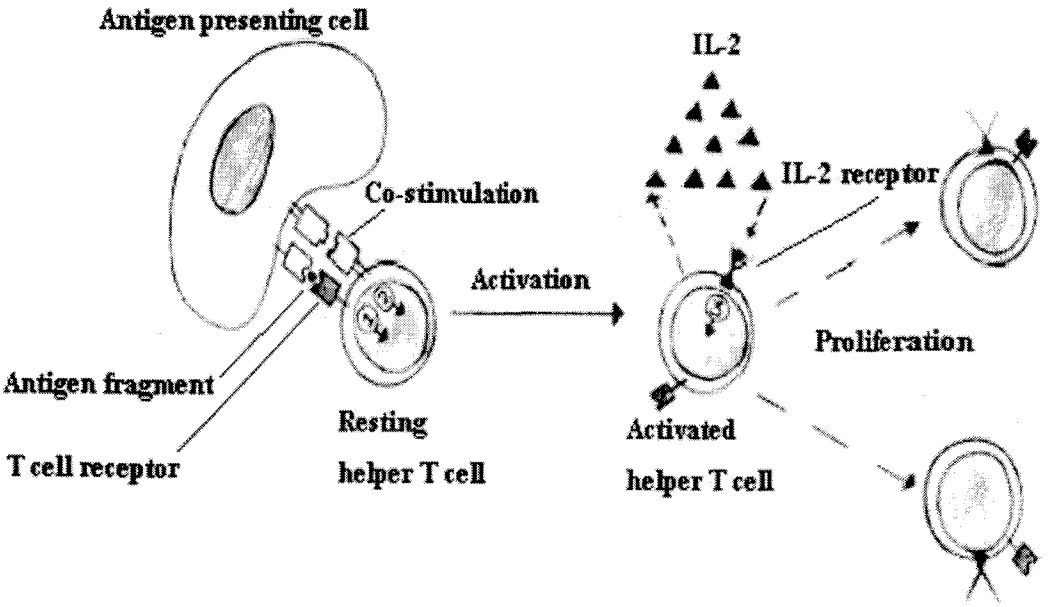


Figure 1.3: T cell proliferation by IL-2*

*Figure modified from Ref. [221].

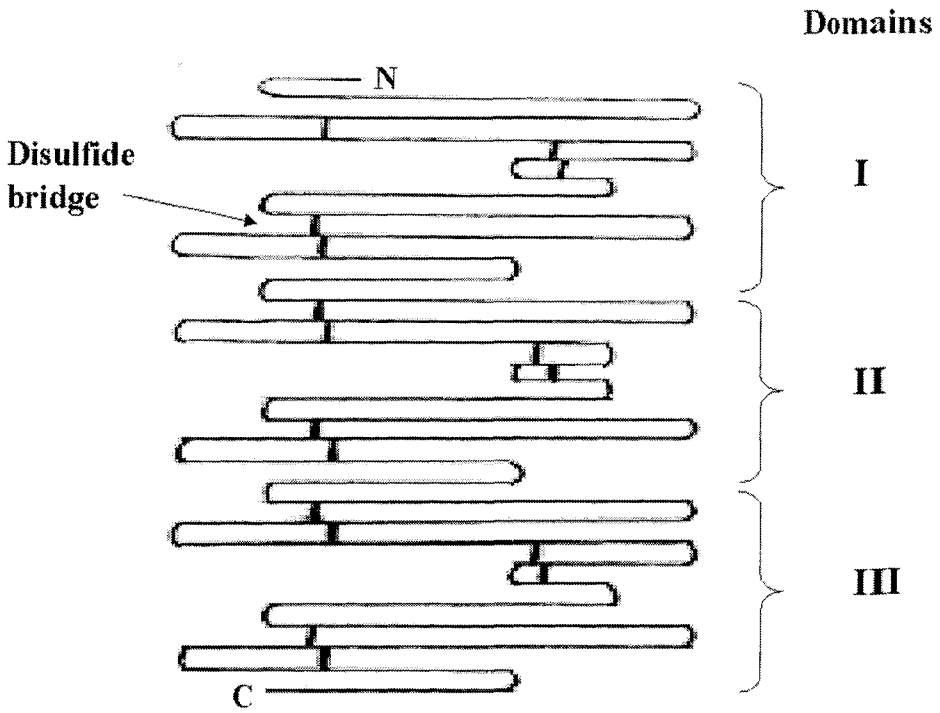


Figure 1.4: Scheme of HSA showing various domains*

*Figure modified from Ref. [196].

Chapter 2

Block copolymer nanoparticles of methoxy polyethylene oxide–*block*-poly (D, L-lactide) for the delivery of interleukin-2

2.1 Introduction

In the context of drug delivery, drug carriers play an important role. The ideal situation for effective therapy is that the drug acts at the target site and have high specificity and activity without causing toxicity. For this purpose, researchers have investigated many drug carriers.

Block copolymers having amphiphilic segments can form core-shell type nanoparticle structure in aqueous milieu [222]. The hydrophobic segment forms the hydrophobic core of the structure and the hydrophilic segment covers the core like a shell. The structurally separated segments of the block copolymer can distinctly share the functions that are required for drug carriers. The outer shell is responsible for interactions with biocomponents such as proteins and cells. These interactions may determine pharmacokinetic behavior and biodistribution of drugs [181]. The inner core serves as a microenvironment for the incorporation of therapeutic molecules. The drug may also be chemically attached to the core-forming block [186]. In this context, we have looked into the possibility of encapsulating an immunostimulatory molecule interleukin-2 inside the core of the particles. Core-shell type nanoparticles of methoxy polyethylene oxide – *block* - poly (D, L – lactide) were chosen as potential carrier for this purpose due to the biocompatibility and biodegradability of MePEO and PDLLA blocks and their distinct functionalities. PEO is known as an inert synthetic polymer in living systems and is used in protein modification to decrease RES uptake and to prolong the half-life in blood. Accordingly, the outer shell, which is composed of PEO chains, is considered to contribute to the stable circulation of particles in blood

[181]. Polyesters such as PDLLA have been widely used in biomedical applications such as sustained drug delivery systems and implants for orthopedic devices. PDLLA is amorphous compared to poly (L-lactic acid) (PLLA) and hence the degradation rate of PDLLA is faster than that of PLLA [223]. Thus, the particles made from PDLLA may be free of toxicities resulting from long-term accumulation.

IL-2 is a cytokine produced by T cells, which plays a prominent role in immunological events. It is being investigated for its therapeutic effects in various cancers and immunotherapy. The primary concern with the use of high dose IL-2 is its toxic side effects. One approach to improve the therapeutic index of IL-2 is to encapsulate the cytokine in a drug carrier so that it accumulates preferentially in the tumor by passive targeting.

Polymeric micelles are known avoid uptake by RES and preferentially diffuse through leaky vasculature of tumor [186]. Tumor specific delivery of IL-2 by polymeric micelles may provide sustained immune stimulation at the tumor site and reduce IL-2 side effects in other tissues. Besides, polymeric micelles can easily be sterilized by filtration [183, 222].

2.2 Materials

Recombinant IL-2 was kindly provided by Biomira Inc (Edmonton, Alberta, Canada). The storage condition for IL-2 was 2-8° C and the buffer system was 0.05 % acetic acid, 5 mg/mL mannitol. Methoxy poly(ethylene oxide) (MePEO), 3,6-dimethyl-1,4-dioxane-2, 5-dione (DL- lactide), and chloroform-d

were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Stannous octate was obtained from ICN Biomedicals Inc (CA, USA). Distilled water was used throughout the study. All other reagents were of analytical grade.

2.3 Methods

2.3.1 Block copolymer synthesis

A ring opening polymerization was employed to synthesize the copolymer of MePEO-*b*-PDLLA. MePEO (MW = 5000 g/mol) was used as initiator (I) and stannous octate as catalyst. D, L – lactide (monomer, Mo; 2.5 g) was first melted in a glass ampoule. MePEO (2.5 g, M/I = 34.7) was weighed into the ampoule and then stannous octate (0.5 % w/w) was added. Nitrogen was purged into the ampoule for 5 min. The glass ampoule was then heat sealed under vacuum using flame torch. The reaction was carried out at 140° C for 3 h. The MW of PDLLA was determined by proton nuclear magnetic resonance (^{1}H-NMR) spectrum at 300 MHz using chloroform-d as solvent. The polymer MW and MW distribution was estimated using gel permeation chromatography (GPC). GPC measurements were carried out with tetrahydrofuran (THF) as the eluent (1 mL/min) using a 4.6 × 300 mm Waters Styragel HT4 column (Waters Inc., Milford, MA). The elution pattern was detected at 35°C by refractive index/light scattering detectors (Model 410, Waters Inc., MA). The calibration curve was prepared using polystyrene standards.

2.3.2 Preparation of core-shell type nanoparticles and their characterization

MePEO-*b*-PDLLA (32.5 mg) was dissolved in 0.5 mL acetone. This solution was added dropwise to 3 mL water while stirring. Acetone was evaporated by stirring it for 3 h at room temperature. Mean diameter and polydispersity index of the particles was determined by dynamic light scattering instrument (3000 HSa Zetasizer Malvern, Malvern Instrument Ltd., UK). Transmission electron microscopy (TEM) was used to investigate the morphology of the particles. An aqueous droplet of dispersion (20 μL) with a polymer concentration of 0.5 mg/mL was placed on a copper-coated grid. The grid was held horizontally for 20 s to allow the colloidal aggregates to settle. A droplet of 2 % solution of phosphotungstic acid in water was then added to provide the negative stain. After 1 min, the excess fluid was removed by filter paper. The samples were then air-dried and loaded into a Hitachi H 7000 transmission electron microscope. Images were obtained at a magnification of 15,000 times. Apparent diameters of particles were measured based on at least 100 measurements.

2.3.3 Loading of IL-2 in MePEO-b-PDLLA nanoparticles

(a) Co-solvent evaporation method

MePEO-*b*-PDLLA (30 mg) was dissolved in 0.5 mL acetone. IL-2 (150 μg, 550 μg/mL) was added to it. Under constant stirring this solution was added dropwise to 3 mL water. Acetone was evaporated by stirring it for 3 h at room temperature. 100 μL of sample was taken and analyzed for free IL-2 by injecting

into size exclusion chromatography (SEC) column (Protein Pak 300 SW column) equilibrated with 0.1 M potassium phosphate monobasic, pH 7.0 at 280 nm. The elution was carried out at a flow rate of 0.8 mL/min. The column was calibrated with MW standards: Blue dextran (2000 kDa), HSA (66 kDa), ovalbumin (44 kDa), trypsin inhibitor (20 kDa), RNase A (14 kDa), and thymidine (242 Da). The void volume of the column was 5.0 mL and total volume was 13.0 mL.

(b) Double emulsion method

MePEO-*b*-PDLLA (100 mg) was dissolved in 1 mL methylene chloride. Aqueous solution of IL-2 (55 μg, 550 μg/mL) was added to it and sonicated (15 s). 8 mL of 5 % polyvinyl alcohol (PVA) solution was added to this emulsion and sonicated (15 s) to form secondary emulsion. This emulsion was added to 17 mL of 1 % PVA solution and stirred for 3 h to eliminate the solvent. The particles were separated by centrifugation at 27,000 × *g* for 30 min. The loading of IL-2 in nanoparticles was calculated from the difference between the total amount used to prepare the formulation and the amount non-encapsulated in supernatant by measuring the tryptophan fluorescence at excitation wavelength of 290 nm.

2.4 Results and discussion

2.4.1 Block copolymer synthesis

Methoxy poly(ethylene oxide)-block-poly(D, L-lactide) was synthesized by ring opening polymerization using MePEO as initiator and stannous octate as catalyst. The scheme of synthesis is shown in figure 2.1. It has been suggested

that the hydroxyl group of MePEO is responsible for initiating the ring opening polymerization [224]. In the reaction, the lactide ring opens and inserts into the initiation site to allow the growth of the polylactide chain resulting in a diblock copolymer of polylactide and methoxy polyethylene oxide. The tin atom of stannous octate coordinates with the carbonyl oxygen atom of lactone. Due to this, the carbonyl carbon atom becomes more positive resulting in an increased susceptibility to nucleophilic attack by a hydroxyl group [225]. The ^{1}H-NMR spectrum of the copolymer is shown in figure 2.2. The peaks of both PDLLA and MePEO were detected in deuterochloroform ($CDCl_3$), which is a good solvent for both PDLLA and MePEO. The peaks at δ = 3.6-3.7 ppm corresponds to methylene protons of MePEO and those at δ = 5.1-5.3 ppm corresponds to methine protons of PDLLA. The MW of PDLLA calculated by comparing the peak intensity of methylene protons of MePEO (assuming a MW of 5000 g/mol) to that of methine protons of PDLLA was 4692 g/mol. The MW of copolymer was determined by GPC. The number-average molecular weight (M_n) and polydispersity of the polymer (M_w/M_n) obtained were 17570 g/mol and 1.008 respectively as shown in table 2.1.

2.4.2 Preparation of core-shell type nanoparticles and characterization

The mean diameter and polydispersity of the particles were measured by dynamic light scattering (DLS). Monodisperse particles were formed with a mean diameter of 67 nm and a polydispersity index of 0.16 (figure 2.3a). The morphology of the particles was assessed by TEM. Figure 2.4 shows that shape of

the particles was spherical and no aggregation was observed. The particles had an approximate size of 66 nm, which was comparable to the size obtained from DLS measurement.

2.4.3 Loading of IL-2 in MePEO-b-PDLLA nanoparticles

(a) Co-solvent evaporation method

The scheme of loading of a drug in amphiphilic block copolymer nanoparticle by co-solvent evaporation is shown in figure 2.5. For assessing the encapsulation of IL-2 in MePEO-*b*-PDLLA nanoparticles, SEC was used. The chromatograms of (a) empty nanoparticles at 220 nm and (b) soluble IL-2 and MePEO-*b*-PDLLA + IL-2 at 280 nm are shown in figure 2.6. There was no peak observed for encapsulated IL-2 in the particles. The area under the chromatogram curve for MePEO-*b*-PDLLA + IL-2 was almost similar to the soluble IL-2 (table 2.2). Moreover, the mean diameter of IL-2 loaded particles did not differ from the empty nanoparticles (figure 2.3b). Thus, there was no significant loading of IL-2 inside the core of the particles. This may be due to the lack of partitioning of IL-2 into the hydrophobic polymer phase. The large size and MW of the protein might have limited its incorporation into the small cargo space in the core of particles. A less loading of moderately water soluble drugs can be regarded as one of the limitation of core-shell type nanoparticle systems. The compatibility between nanoparticle core and the drug, the hydrophobic/hydrophilic ratio of the polymer and molecular volume of the drug can influence efficiency of drug encapsulation. Modifying the block composition of block copolymer might improve entrapment

of IL-2. For example, a higher loading may be obtained by decreasing PDLLA/MePEO ratio in the block copolymer. Also, IL-2 conjugated to the polymer may provide a better incorporation. This may be achieved by preparing nanoparticles with reactive aldehyde group at the end of PEG and subsequent quenching of aldehyde group with cysteine. The particles may then be used to conjugate IL-2 by disulfide linkage.

(b) Double emulsion method

In order to quantify IL-2, the particles were separated and non-encapsulated IL-2 was measured by fluorescence intensity. IL-2 was detected based on the fluorescence of the single tryptophan residue within the protein molecule. Analysis of IL-2 in nanoparticles could not be performed due to high background reading for IL-2. This was due to the interference by mannitol in IL-2 solution (5 mg/mL), which was added for storage purposes. Thus the analysis was not feasible.

2.5 Conclusions

MePEO-*b*-PDLLA block copolymer could be synthesized by a ring opening polymerization reaction. This block copolymer self assembled to form core-shell type nanoparticles with a mean diameter less than 100 nm and a narrow polydispersity index. The particles were uniform and spherical in shape. They were used for assessing the encapsulation of IL-2 inside their core. The results suggest that there was no significant loading of IL-2 within the particles. In conclusion, the current study did not provide evidence of sufficient encapsulation

of the protein to pursue further studies.

Table 2.1. Characteristics of the prepared MePEO-*b*-PDLLA block copolymer

MePEO MW (g/mol)	PDLLA MW [a] (g/mol)	Mo/I [b]	MePEO-*b*-PDLLA M_n [c] (g/mol)	MePEO-*b*-PDLLA M_n [d] (g/mol)	MePEO-*b*-PDLLA polydispersity (M_w/M_n) [d]
5000	5000	34.7	9692	17,570	1.008

[a] Theoretical molecular weight
[b] Monomer to initiator ratio
[c] M_n is number average molecular weight; determined by ^{1}H-NMR
[d] M_w is weight average molecular weight; determined by GPC

Table 2.2. Area under the size exclusion chromatogram curve for soluble IL-2 and MePEO-*b*-PDLLA + IL-2 detected at 280 nm using Protein Pak 300 SW column with retention times of 14.3 and 14.5 min respectively.

Type of sample	Area under the chromatogram curve (μV.min)
Soluble IL-2	10469
MePEO-*b*-PDLLA + IL-2	9551

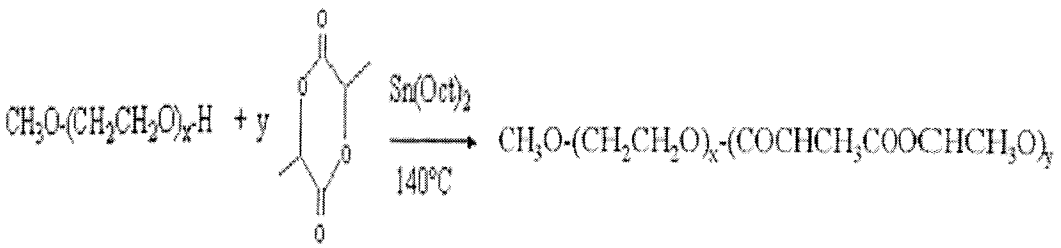


Figure 2.1 Scheme of synthesis of MePEO-*b*-PDLLA block copolymer

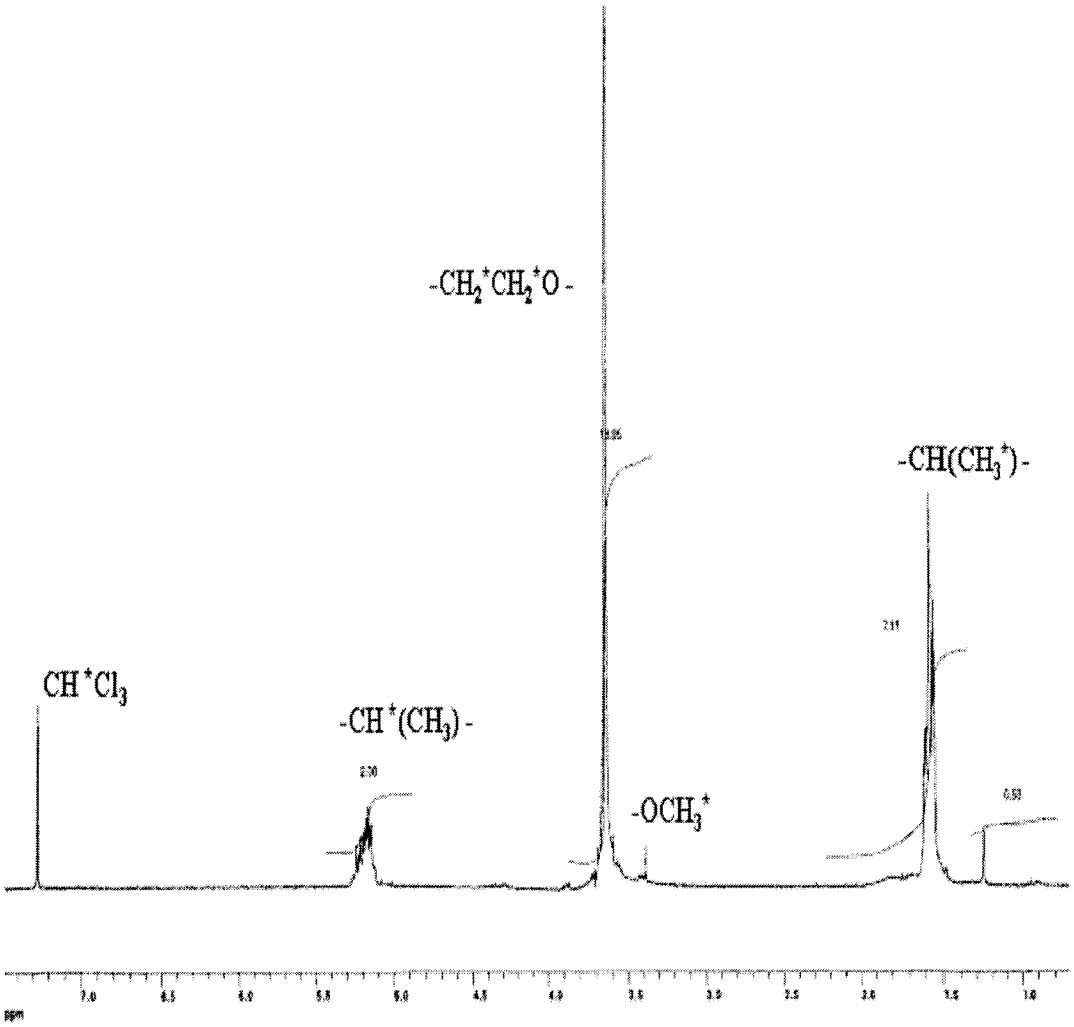


Figure 2.2. ^{1}H-NMR spectrum of MePEO-*b*-PDLLA block copolymer in $CDCl_3$ at 300 MHz; * denotes the different protons giving shifts.

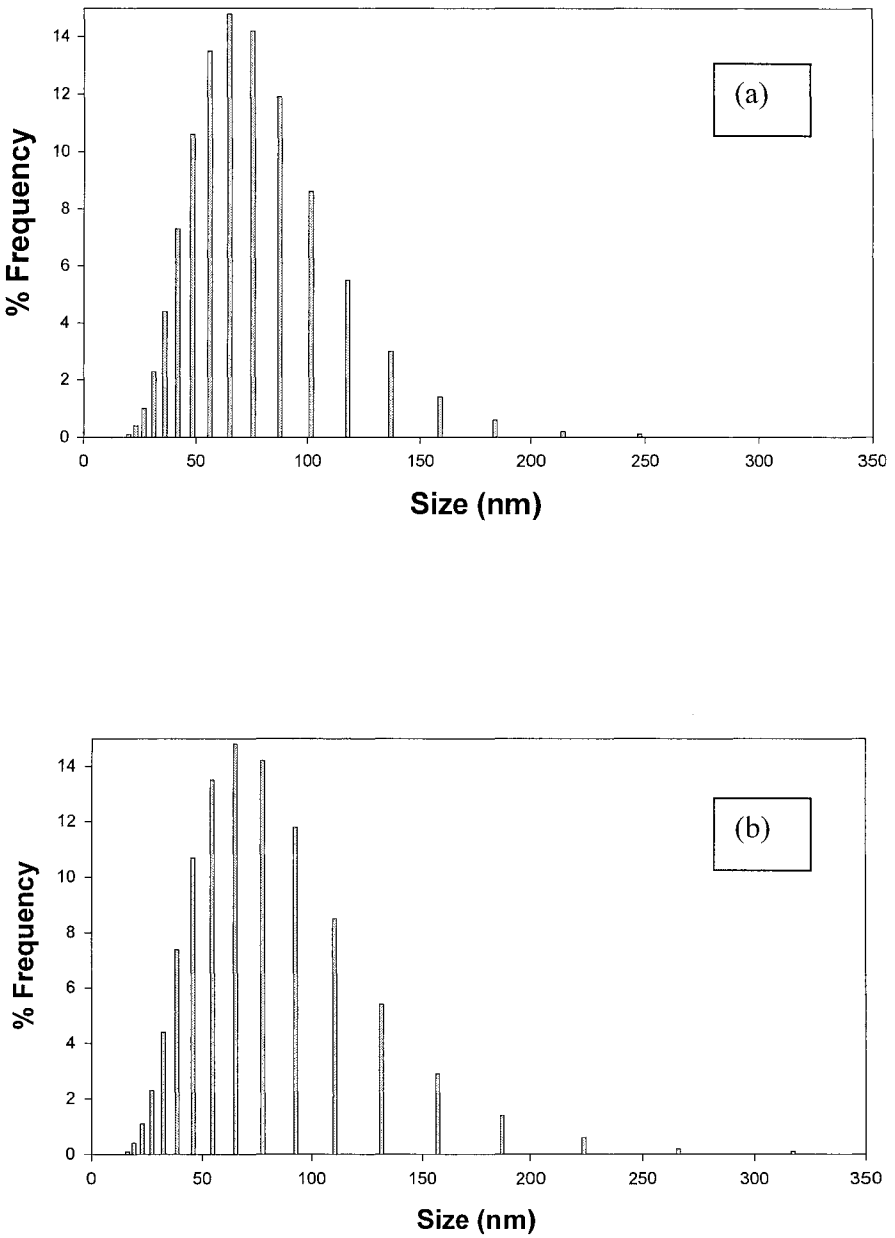


Figure 2.3. Size distributions (intensity-mode analysis) of (a) empty MePEO-*b*-PDLLA block copolymer nanoparticles and (b) MePEO-*b*-PDLLA particles after IL-2 loading as measured by dynamic light scattering instrument (Zetasizer 3000).

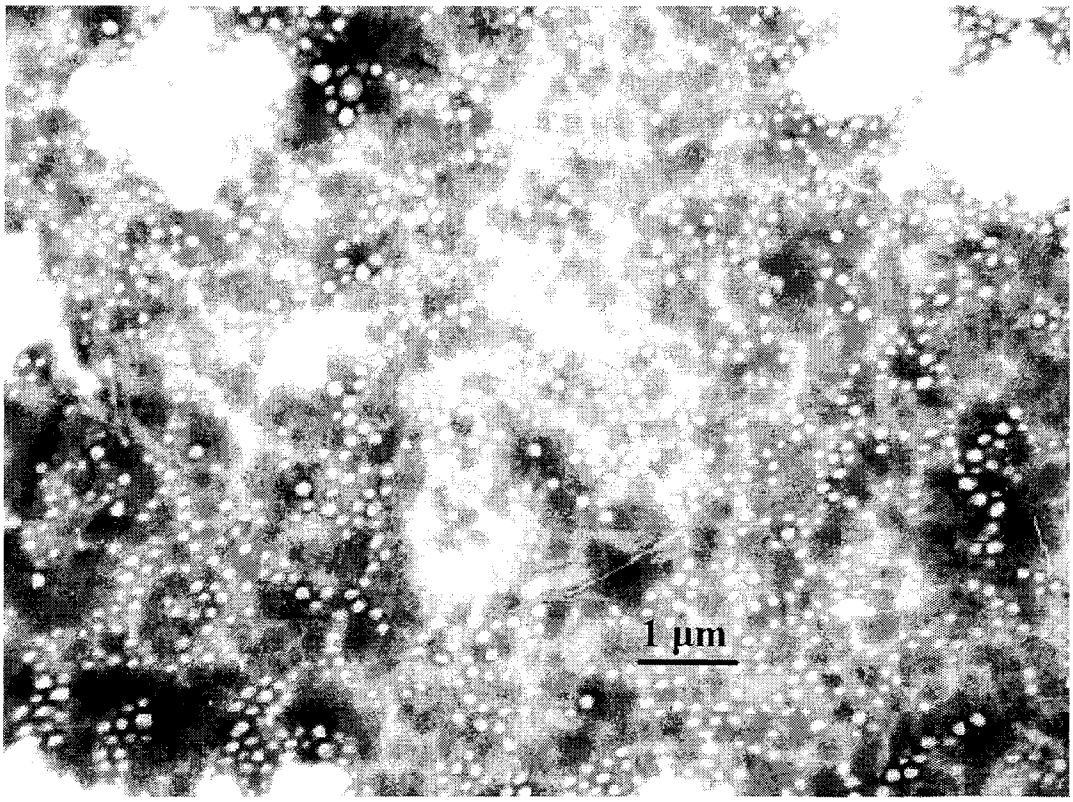


Figure 2.4. TEM image of MePEO–*b*-PDLLA block copolymer nanoparticles (magnification 15,000)

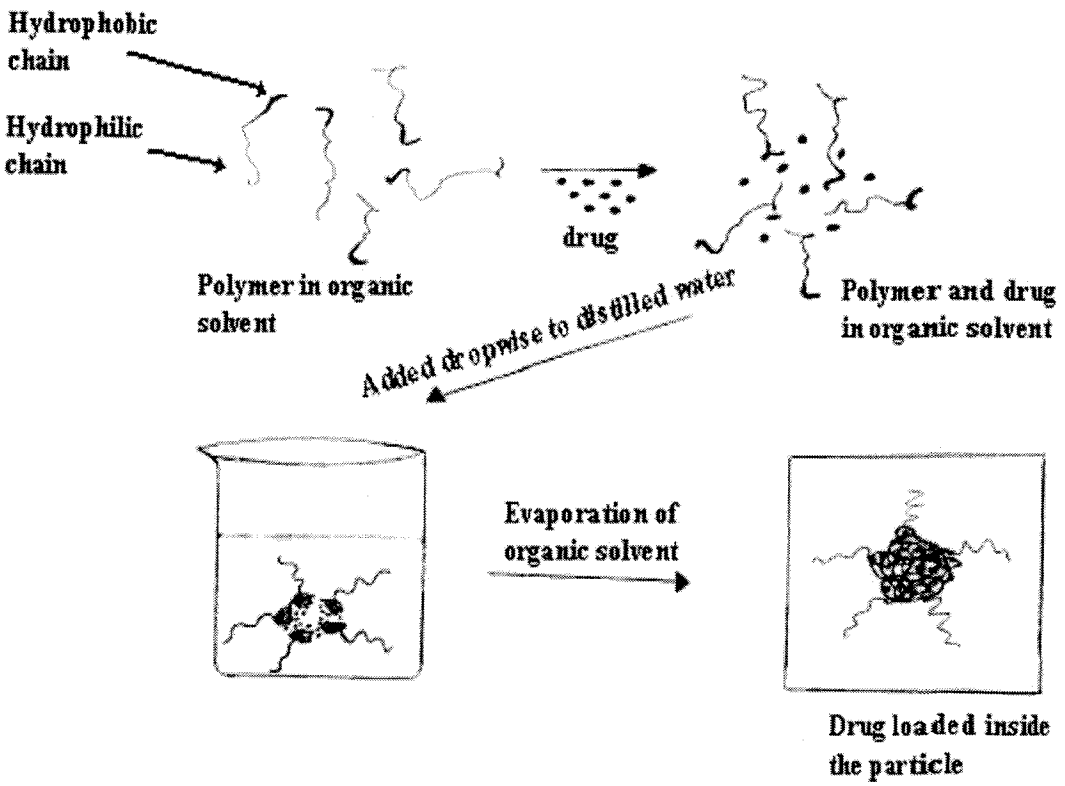
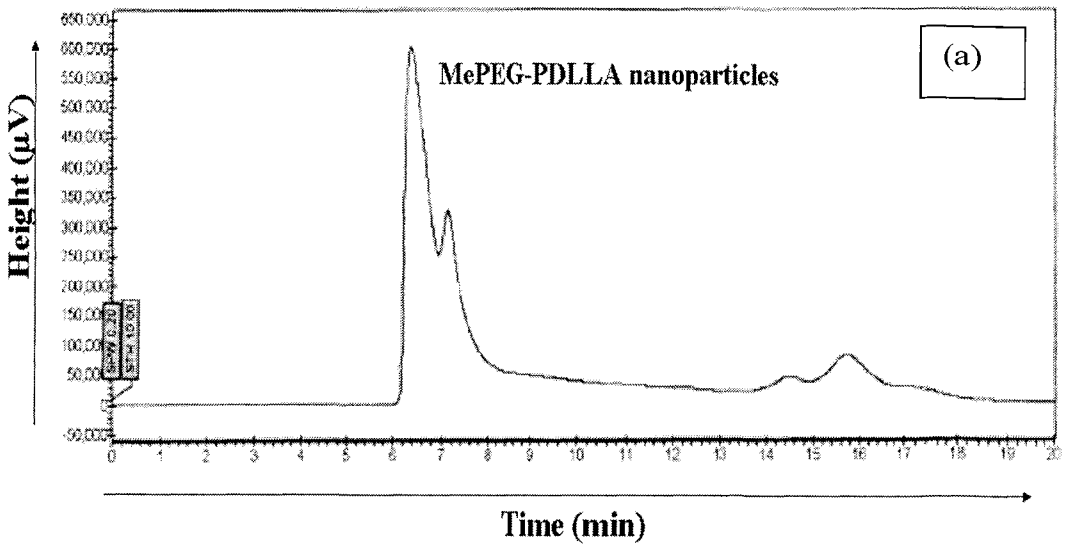


Figure 2.5 Scheme of loading of a drug in amphiphilic block copolymer nanoparticle by co-solvent evaporation method*.

*Figure modified from Ref. [183].



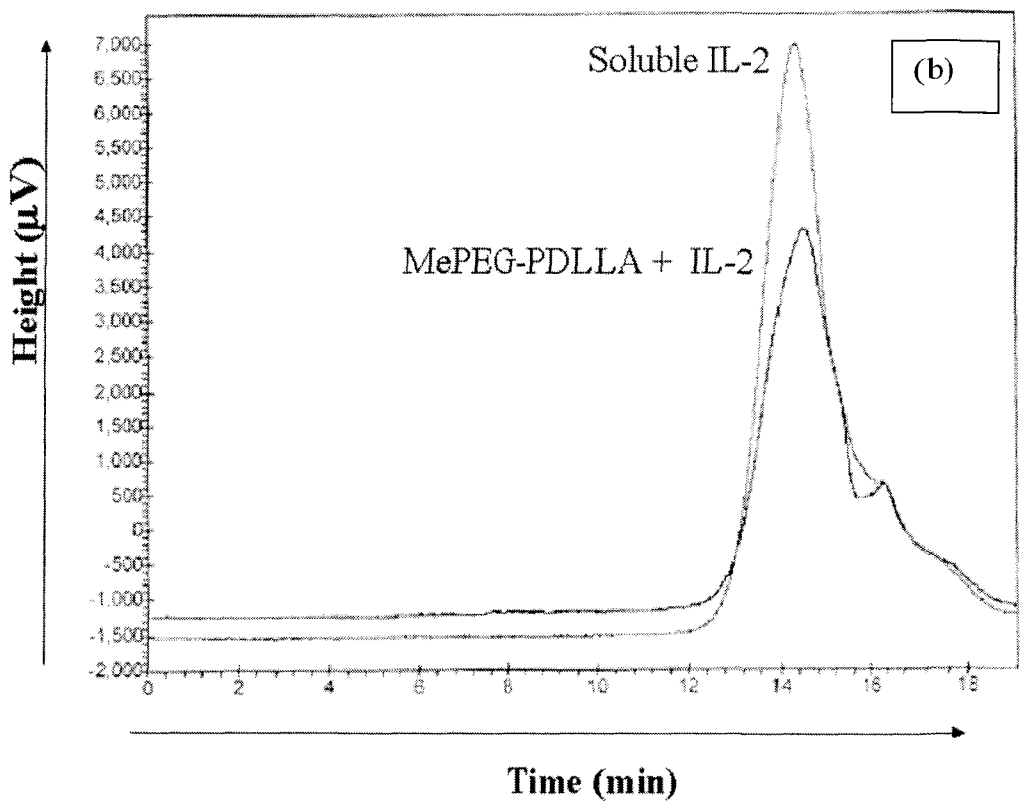


Figure 2.6. Size exclusion chromatograms of (a) empty MePEO-*b*-PDLLA block copolymer nanoparticles detected at 220 nm and (b) soluble IL-2 and MePEO-*b*-PDLLA + IL-2 at 280 nm using Protein Pak 300 SW column equilibrated with 0.1 M potassium phosphate monobasic (pH 7.0).

Chapter 3

Human serum albumin nanoparticles for the delivery of interleukin-2

3.1 Introduction

Colloidal drug carrier systems are advantageous for modifying biodistribution, enhancing cellular uptake and limiting the toxic side effects of drugs [226-228]. When a drug is bound to a polymeric carrier by covalent linkage, such a conjugate can potentially maintain constant delivery of drug to the body over a prolonged period of time. In the present study, we have investigated the chemical conjugation of IL-2 to HSA nanoparticles.

HSA nanoparticles were chosen as colloidal carrier system due to a number of attractive features. HSA is a biocompatible natural macromolecule that has been used in several protein drug formulations. HSA appears to prevent aggregation and enhance the solubility and stability of IL-2 [220]. HSA offers several target sites for covalent linkage of drugs like the ε-amino groups of lysine, carboxyl groups of asparatic and glutamic acid, hydroxyl groups of tyrosine and thiol groups of cysteine. Thus, it has been shown to be amenable to particle engineering and surface modification [202, 228]. It also lacks toxicity and is readily available.

Weber *et al* has shown that it is possible to introduce thiol groups on the surface of HSA nanoparticles using dithiothreitol (DTT) [202]. Moreover, IL-2 has a free cysteine residue at amino acid position 125 [14]. We devised a method of conjugating IL-2 to HSA nanoparticles through formation of disulfide bonds between the free thiol groups generated on the nanoparticles using DTT and free thiol of cysteine-125 of IL-2.

3.2 Materials

Recombinant interleukin-2 (IL-2) and cytotoxic T lymphocyte line (CTLL-2) cells were kindly provided by Biomira Inc (Edmonton, Alberta, Canada). HSA, glutaraldehyde solution (25 %) and L-cysteine were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Iodine-125 was purchased from Amersham Biosciences (Quebec, Canada). Iodogen, 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) and DTT were purchased from Pierce Biotechnology (Rockford, IL, USA). RPMI 1640 (Roswell Park Memorial Institute) medium and other cell culture supplements were provided by Gibco Invitrogen Corporation (Ontario, Canada). Distilled water (Milli-Q system, Millipore, USA) was used throughout the study. All other reagents were of analytical grade and used as received.

3.3 Methods:

3.3.1 Radioiodination of IL-2

A 13 x 75 mm glass test tube was plated with 10 μg Iodogen solution in chloroform. The solvent was evaporated by running a steady stream of nitrogen gas into the tube until fully dry. To another test tube, 20 μL phosphate buffer (0.5 M, pH = 7.1) and 5 μL ^{125}I (0.5 mCi) were added together. IL-2 (30 μg) in phosphate buffered saline (PBS) was added to the Iodogen tube. The phosphate buffer - ^{125}I mixture was then transferred to the Iodogen tube. The sample was incubated for 30 min at room temperature. The sample was removed from Iodogen tube and placed in a clean glass tube. To stop the reaction 70 μL sodium iodide

(NaI; 1 M) was added. The free iodine was separated using a Sephadex PD10 column (MW cut-off = 5 kDa; Amersham Biosciences, Quebec, Canada).

3.3.2 Preparation of HSA nanoparticles

HSA (200 mg) was dissolved in 2.0 mL double distilled water. Under constant stirring, desolvation of the 10 % HSA solution was achieved by drop wise addition of 8.0 mL ethanol. After 10 minutes of stirring, 20 μL glutaraldehyde solution (25 %) was added to cross-link the particles. After 4 h of stirring, the nanoparticles were purified 3 times by centrifugation and redispersion in purified water. Mean size and polydispersity index of the particles were determined by dynamic light scattering instrument (Zetasizer 3000, Malvern, UK).

3.3.3 Introduction of thiol groups with DTT

Aqueous solution of DTT (50 μL, 50 mg/mL) was added to nanoparticle suspension (100 μL) and mixed. The samples were incubated for 2 h and purified by centrifugation at 20000 × *g* for 20 min.

3.3.4 IL-2 conjugation to HSA nanoparticles

The purified particles were mixed with 50 μL of radiolabeled IL-2. The samples were incubated in a shaking water bath at 20° C for 12 h and 24 h. Volume was adjusted to 300 μL and after further shaking for 2 h the samples were centrifuged for 20 min at 20000 × *g*. The nanoparticle pellet and supernatant were

analyzed using gamma counter (1480 Wizard 3", Wallac). Loading efficiency of IL-2 was calculated as:

$$\text{Loading efficiency (\%)} = \frac{\text{IL-2 in nanoparticle pellet}}{\text{Total IL-2 added}} \times 100$$

3.3.5 Trichloroacetic acid (TCA) precipitation of IL-2

The radioiodinated pellet was redispersed in 300 μL of purified water. Aliquots of the dispersion (20 μL) were treated with 20 μL of 1 M NaI. The samples were prepared for TCA precipitation in microcentrifuge tubes as follows: 0.5 mL of a solution of 1 % BSA in PBS was added to Eppendorf centrifuge tubes. 2 μL of the above treatment was added to the cold BSA. 0.5 mL of 20 % TCA was added and vortexed well. The samples were cooled for 3 min in ice and then centrifuged for 3 min at 20000 × *g*. The supernatants were transferred to different centrifuge tubes. The tubes were placed into counting vials and the count rate was recorded in gamma counter.

3.3.6 Quantification of thiol groups in nanoparticles

An aliquot of HSA nanoparticle suspension (100 μL, 50 mg/mL) was mixed with volumes of 10, 20, 50, 100, 200, 500 μL of aqueous solution of DTT (50 mg/mL). The volume was adjusted with distilled water. The samples were incubated for 2 h and purified by centrifugation (20000 × *g* for 20 min). L-cysteine standards (0–5 mM) were prepared and the number of thiol groups was quantitated by comparison to the standard values. The nanoparticles were diluted with distilled water so that the sample applied to the assay reaction has a thiol

concentration in the working range of the standard curve. HSA nanoparticles (100 μL) were mixed with 1365 μL of reaction buffer (0.1 M sodium phosphate, pH 8.0, containing 1 mM Ethylenediamine tetra acetic acid, EDTA) and 35 μL of 0.4 % Ellman's reagent solution. After 15 min incubation, the particles were separated by centrifugation (20000 × *g* for 20 min). The supernatant was spectrophotometrically assayed for 2-nitro-5-thiobenzoic acid (TNB) at 412 nm. The samples were prepared for thiol quantification as follows: (a) Plain HSA nanoparticles, (b) HSA nanoparticles reduced by DTT, (c) HSA nanoparticles loaded with IL-2 and (d) HSA nanoparticles loaded with IL-2 and reduced by DTT.

3.3.7 Determination of the *in vitro* activity of IL-2 using the CTLL-2 assay

The CTLL-2 cells were grown in the growth media (RPMI 1640 440 mL, fetal bovine serum 50 mL, MEM-sodium pyruvate 5 mL, 2-mercaptoethanol 100 μl, Gentamycin 100 μl). The tissue culture was divided every second day and the cell population was kept below 1×10^5 cells/mL with 10 IU IL-2/mL. At the time of the assay the cells were more than 95 % viable as estimated by trypan blue dye exclusion test. Before the cells were seeded into the 96-well plates they were washed three times with assay media to remove any free IL-2 from the culture. Cells then were seeded into each well of the 96-well plate (1×10^4 cells/ well). Test samples of IL-2 in concentrations: 2, 4, 8, and 16 ng loaded on 500 μg HSA nanoparticles (each in triplicate) were added to the wells. Each plate also contained wells with soluble IL-2, empty nanoparticles and untreated cells as

controls. After incubation for either 24 hours or 4 days at 37°C, the cells were incubated with [^{3}H]-thymidine (1 μCi in 50 μL of medium/ well) for 24 hours. The cells were then harvested (Harvester 96, Tomtec, Hamden, USA) on filters and treated with Meltilex A (Wallac, Turku, Finland) and counted (1450 Microbeta Trilux, Wallac, Turku, Finland).

3.4 Results

3.4.1 Radioiodination of IL-2

IL-2 was radiolabeled by Iodogen plating technique. 40 fractions of the prepared sample were collected in different tubes after passing through PD10 column (MW cut-off = 5 kDa). The high MW of radiolabeled IL-2 allows it to pass freely through the column. Labeled IL-2 eluted first followed by free iodine as shown in figure 3.1.

3.4.2 Characterization of HSA nanoparticles

HSA nanoparticles were prepared by an adapted procedure based on a previously reported method by Marty et al [200]. Dynamic light scattering gave evidence for the formation of nanoparticles. The empty particles showed a mean diameter of 206 nm and a polydispersity index of 0.04 as shown in figure 3.2A.

3.4.3 IL-2 conjugation to HSA nanoparticles

Loading efficiency of IL-2 in HSA nanoparticles was determined using

radiolabeled IL-2. Most of the radioactivity was detected from the nanoparticle pellet. TCA precipitation of the nanoparticle pellet was done to determine the percent bound radioiodine in the sample. The activity from TCA precipitates showed that greater than 96 % of the radioactive iodine was bound to IL-2. After 12 and 24 h of incubation, IL-2 conjugation efficiency was 92 and 90 %, respectively (figure 3.4). The IL-2 conjugated particles showed an increase in mean diameter from 206 to 237 nm (figure 3.2B).

3.4.4 Quantification of thiol groups in nanoparticles

The number of free thiols available for disulfide bond formation in HSA nanoparticle surface before and after IL-2 conjugation was determined by 5, 5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent) [229]. The results of the thiol group determinations after treatment with raising amounts of DTT are shown in figure 3.5.

An increase in the concentration of DTT from 44 to 2173 mol per mol HSA resulted in an increase in the number of free thiol groups. At the highest concentration, the free thiol groups were calculated as 9.43 ± 0.05 mol per mol HSA. To assess the extent of IL-2 conjugation by disulfide linkage, HSA nanoparticles reduced by DTT (20 μL, 50 mg/mL) were incubated with 35.6 μg of IL-2 for 12 h. The results are shown in figure 3.6. After treatment free thiol groups showed a significant decrease from 6.27 ± 0.14 to 3.99 ± 0.18 mol per mol HSA ($P<0.001$, unpaired students t-test). This indicates a 37 ± 1.5 % reduction in the content of free thiol group per mol HSA. The decrease in the number of free

thiol groups after treatment with IL-2 reflects the formation of disulfide bonds between IL-2 and HSA. The IL-2 conjugated HSA nanoparticles were treated with DTT to break the formed disulfide bonds. After DTT treatment a significant increase in the free thiol group content from 3.99 ± 0.18 to 5.54 ± 0.2 mol thiol per mol HSA was observed ($P<0.001$, unpaired students t-test).

The degree of decrease in the free thiol group content on HSA during disulfide linkage was dependent on the initial applied level of IL-2 (figure 3.7). When amount of IL-2 was increased from 8.9 to 106.8 μg, a decrease in the number of free thiol groups from 5.2 ± 0.27 to 3.43 ± 0.54 mol per mol HSA was observed. Based on the results, the total decrease of thiol groups was more (13.2×10^{-8} moles) corresponding to the total increase of IL-2 added (0.58×10^{-8} moles). Thus it is expected that all of the added IL-2 was conjugated to the particles.

3.4.5 Determination of the *in vitro* activity of IL-2 using the CTLL-2 assay

The CTLL-2 assay was established to investigate the bioactivity of IL-2 conjugated HSA nanoparticles. Figure 3.8 compares the biological activity of IL-2 conjugated HSA nanoparticles with soluble IL-2 after 24 h culture. The activity of IL-2 conjugated particles was found to be lower than soluble IL-2 during this period. Comparison of bioactivity over a period of 4 days in culture is shown in figure 3.9. At this time, IL-2 conjugated HSA nanoparticles (at IL-2 conjugation levels of 4, 8 and 16ng/500 μg of HSA) showed higher activity than the soluble IL-2. At a lower IL-2/HSA conjugation content (2 ng IL-2/500 μg of HSA), no

significant difference between the bioactivity of soluble and HSA conjugated IL-2 was observed ($p > 0.05$, unpaired students t test).

3.5 Discussion

The radioiodination of proteins usually involves the electrophilic aromatic substitution of radioiodine for the aromatic protons of tyrosine in a protein molecule. Electropositive radioiodine necessary for this reaction has been prepared by using the solid phase reagent, Iodogen, which was first described by Fraker and Speck [230]. Iodogen has a number of advantages over traditional iodinating reagents including its rapid and selective action. It is soluble in chloroform but insoluble in aqueous solutions. This characteristic allows a plating technique to be used in the iodination. After Iodogen plating to the reaction vessel, aqueous solution of IL-2 is added to the reaction vessel followed by radioiodine. The electrophilic species formed in the solid state reaction between iodine and Iodogen then undergo an electrophilic aromatic substitution with the dissolved IL-2.

In the present study, protein nanoparticles were made of HSA. The desolvation of HSA with organic solvents followed by cross-linking with glutaraldehyde is a commonly used method for the preparation of protein nanoparticles [200]. Glutaraldehyde was used at a minimal amount (25 %) to stabilize the particles. Glutaraldehyde forms chemical cross-links by linking the free amino groups on the surface of the HSA nanoparticles [231]. HSA nanoparticles of approximately 200 nm were prepared using this method.

Polydispersity index gave indication of the monodispersity of the particles and absence of aggregated species.

IL-2 was linked to the surface of HSA nanoparticles through disulfide bonds. Disulfide bonds (-S-S-) are covalent bonds formed between sulfur atoms of cysteine residues through oxidation.

$$Cys{-}SH + SH{-}Cys \rightarrow Cys{-}S{-}S{-}Cys$$

Thiol groups (-SH) of cysteine on HSA nanoparticles were used as target site for the covalent linkage of IL-2. One mol of the protein HSA consists of 17 disulfide bonds. It has to be assumed that after the preparation of HSA nanoparticles a certain amount of disulfide linkages of the HSA molecules remains on the surface of the particulate system. Free thiol groups were generated on the nanoparticles by reducing these disulfide bonds with the strong reducing agent, DTT [232]. DTT may also reduce the disulfide bonds inside the particles. The particles were subsequently incubated with IL-2 for formation of a disulfide bond, preferably through free thiol group of cysteine-125 of IL-2 as shown in the scheme (figure 3.3).

The HSA nanoparticulate system was characterized concerning the number of available thiol groups. This was determined using Ellman's reagent. Ellman's reagent reacts with a free thiol to yield a mixed disulfide and TNB. TNB is a "colored" species produced in this reaction, which was quantified. The results of thiol quantification indicate that free thiols were readily available on HSA nanoparticle surface as well as bulk before loading and IL-2 was conjugated by disulfide bond formation not simple adsorption. Further investigations

revealed that IL-2 conjugation on HSA nanoparticles is dose-dependent.

The nanoparticles loaded with IL-2 were subsequently tested in the CTLL-2 bioassay to determine the effect of the covalent linkage on its biological activity. CTLL-2 cells are dependent on IL-2 for their growth and therefore ideal for determining the biological activity of IL-2 *in vitro*. The incorporation of tritiated thymidine into DNA provides a quantitative estimate of IL-2 induced CTLL-2 proliferation [233].

For lower IL-2/HSA loading ratios the differences in CTLL-2 proliferation between the conjugated and free IL-2 were not significant after 24 h or 4 days of incubation. When the ratio of conjugated IL-2 to HSA particles was increased, the cell proliferation generally increased, but the proliferative effect of conjugated IL-2 was usually less than that of soluble IL-2. The study suggests that IL-2 bound on the nanoparticles is capable of engaging IL-2 receptors on the target cell surface and induce clonal expansion of T cells but to a lower extent to free IL-2. Moreover, IL-2 linked to nanoparticles retained its functional activity over the 4 day culture period. Whereas free IL-2 at higher concentration have shown a decrease in its bioactivity after 4 days of incubation (Figure 3.8B). The higher activity with conjugated IL-2 may be due to the slow release of IL-2 from the particles over the 4 day culture period.

IL-2 coupled to polystyrene beads to form a solid matrix has shown to be biologically active [234]. Here, IL-2 was linked to the matrix through carbodiimide bond. The released IL-2 was able to increase the cytotoxicity of rat spleen cells *in vitro*. The release may be due to the gradual dissolution of

aggregated IL-2 bound to the bead matrix. The i.p. injection of IL-2 coupled beads resulted in the *in vivo* activation of nonspecific cytotoxic cells in that compartment. However, this formulation is not safe for human use because polystyrene is not biocompatible.

3.6 Conclusions

In the present study, HSA nanoparticles were used to covalently attach IL-2 through disulfide bond formation. The resulting formulation has shown a reduced but prolonged biological activity in terms of CTLL-2 proliferation when compared to free IL-2.

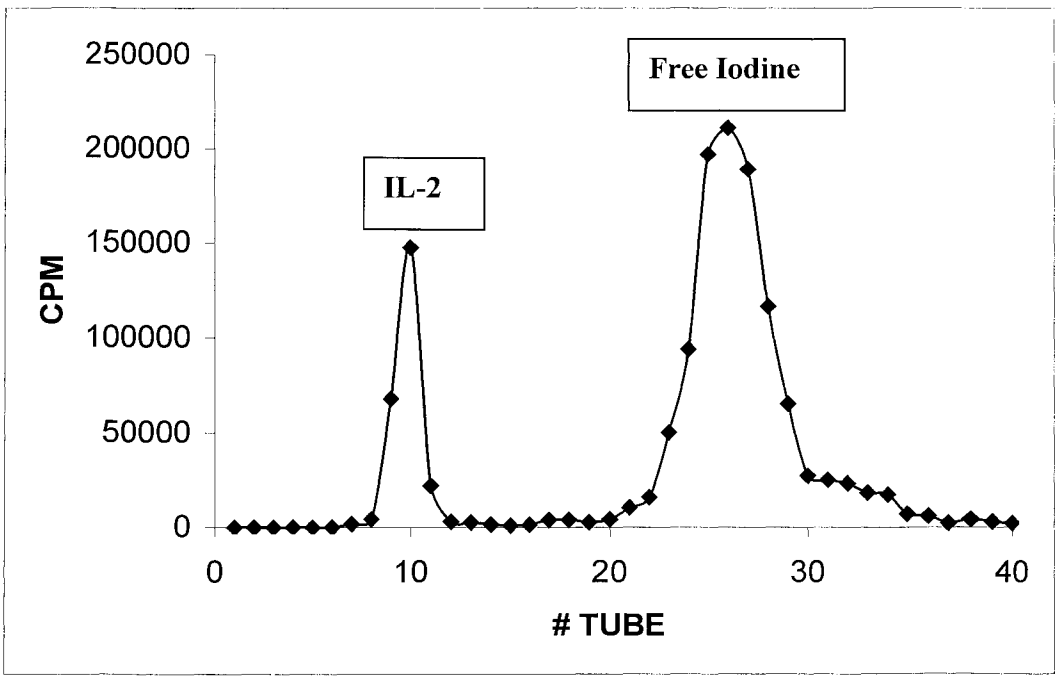


Figure 3.1. Determination of the efficiency of ^{125}I labeling of IL-2 using a prepacked size exclusion column (Sephadex PD10 column).

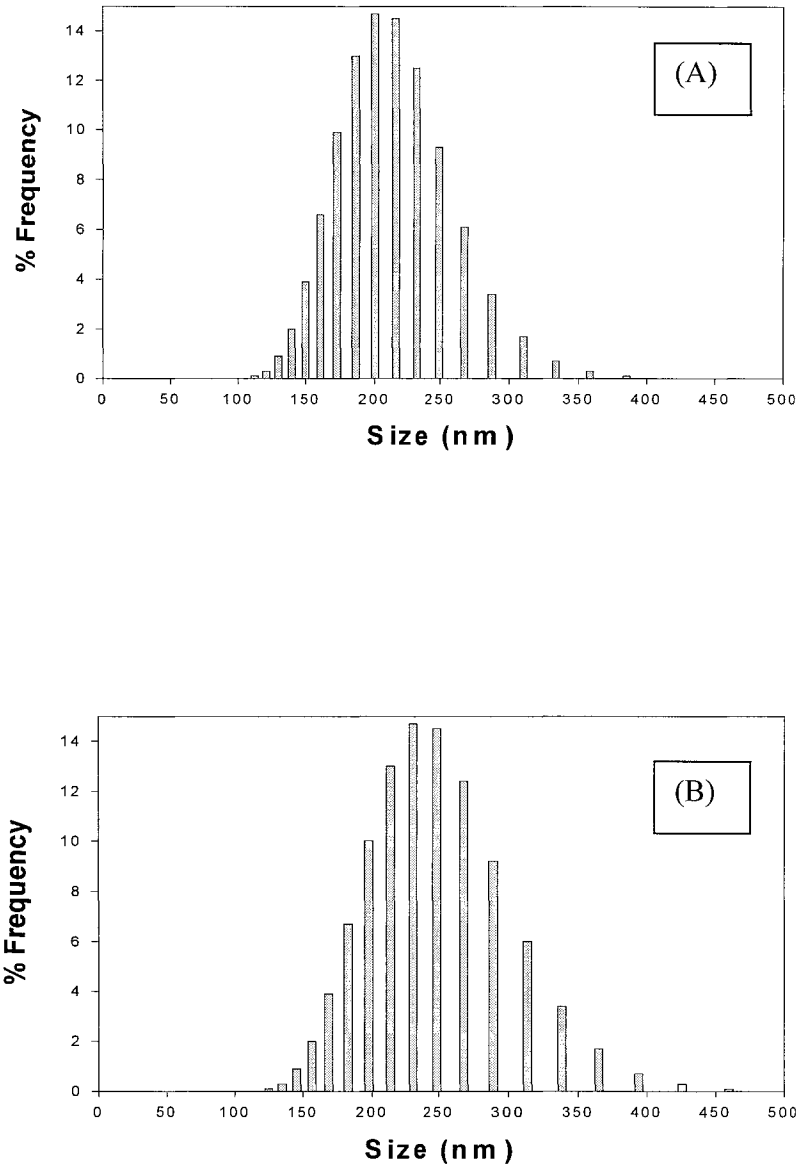


Figure 3.2. Size distributions (intensity-mode analysis) of (A) empty HSA nanoparticles and (B) IL-2 conjugated particles as measured by dynamic light scattering instrument (Zetasizer 3000).

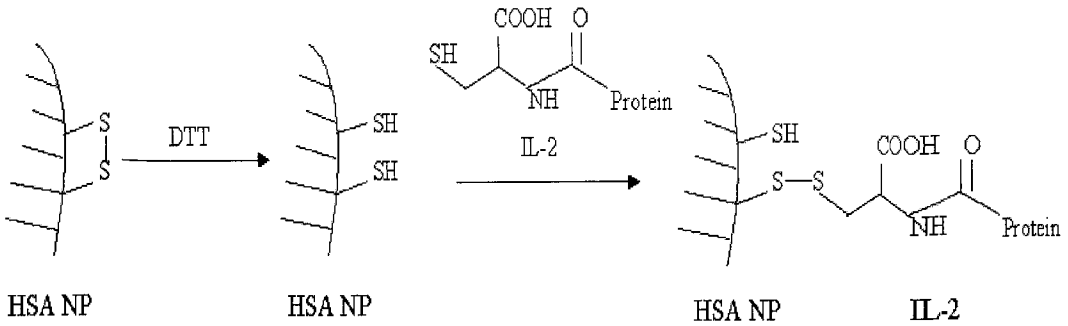


Figure 3.3. Scheme of generation of thiol groups on HSA nanoparticles and subsequent conjugation of IL-2.

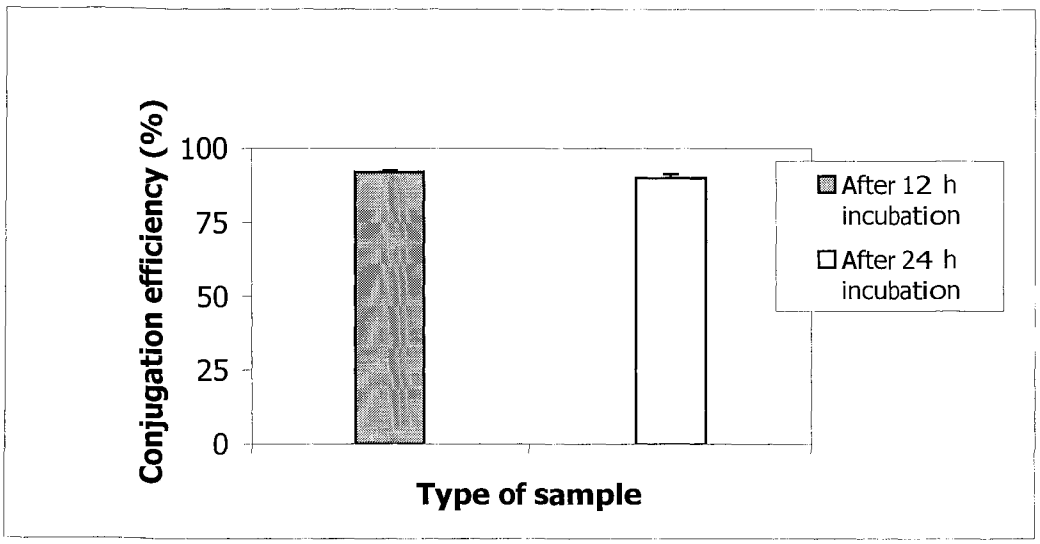


Figure 3.4. Conjugation efficiency of IL-2 to HSA nanoparticles after incubation for 12 and 24 h (mean ± range; n = 2).

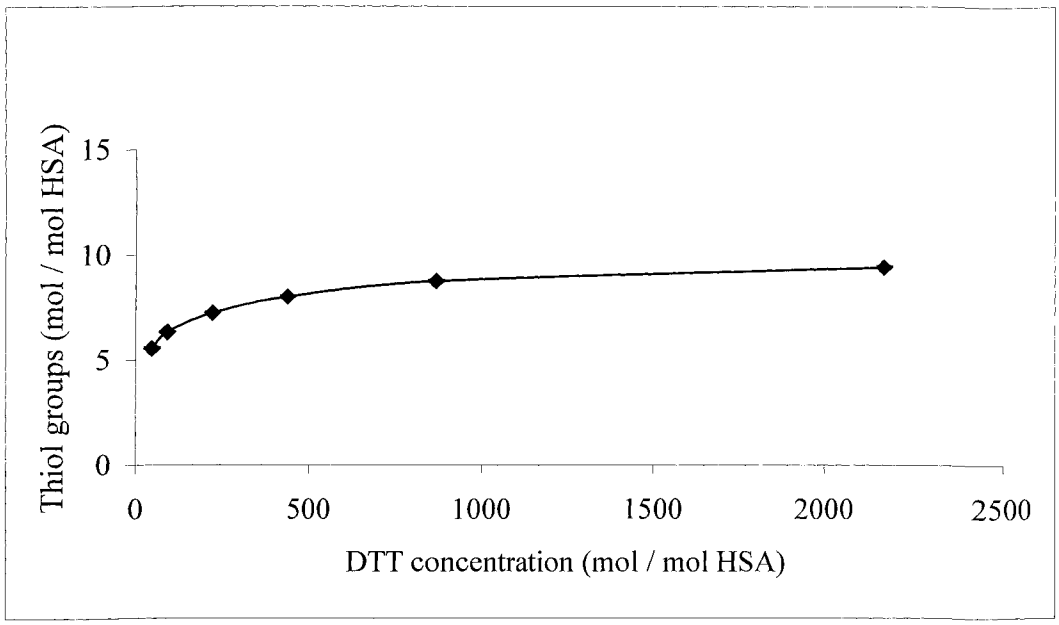


Figure 3.5. Generation of thiol groups using DTT: number of reactive thiol groups introduced per HSA molecule in correlation to the amount of DTT added for HSA disulfide reduction (mean ± range; n = 2).

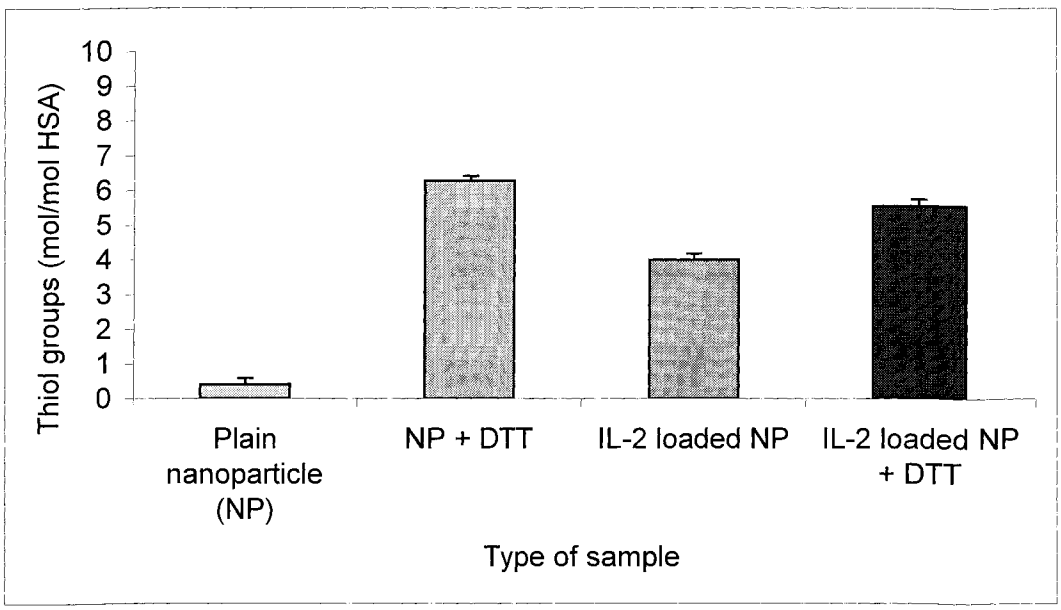


Figure 3.6. Quantification of the free thiol groups on HSA nanoparticle surface before and after treatment with DTT (mean ± S.D; n = 3).

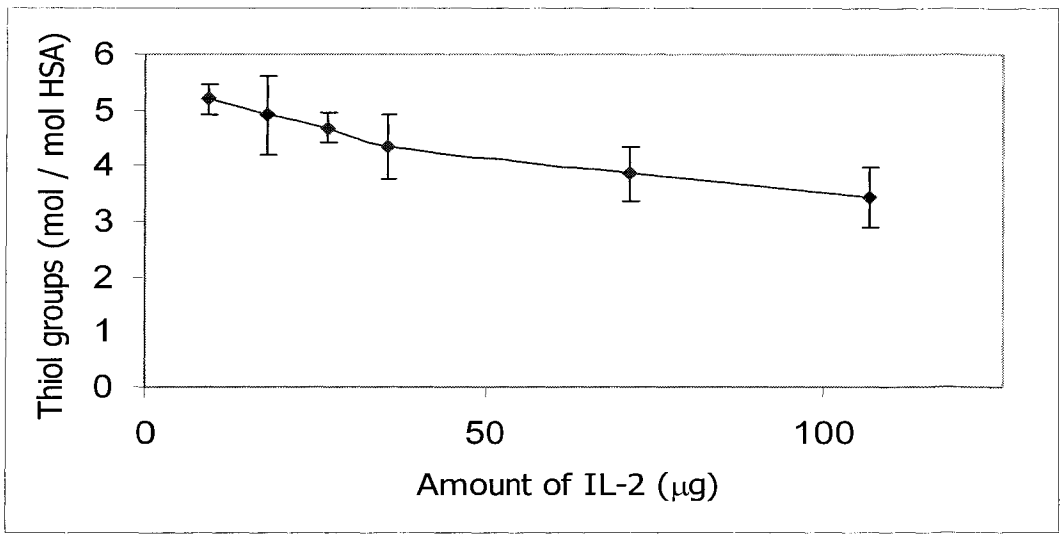


Figure 3.7. Reduction in the quantity of free thiol groups on HSA nanoparticles (5 mg) with raising amounts of IL-2 (mean ± S.D; n = 3).

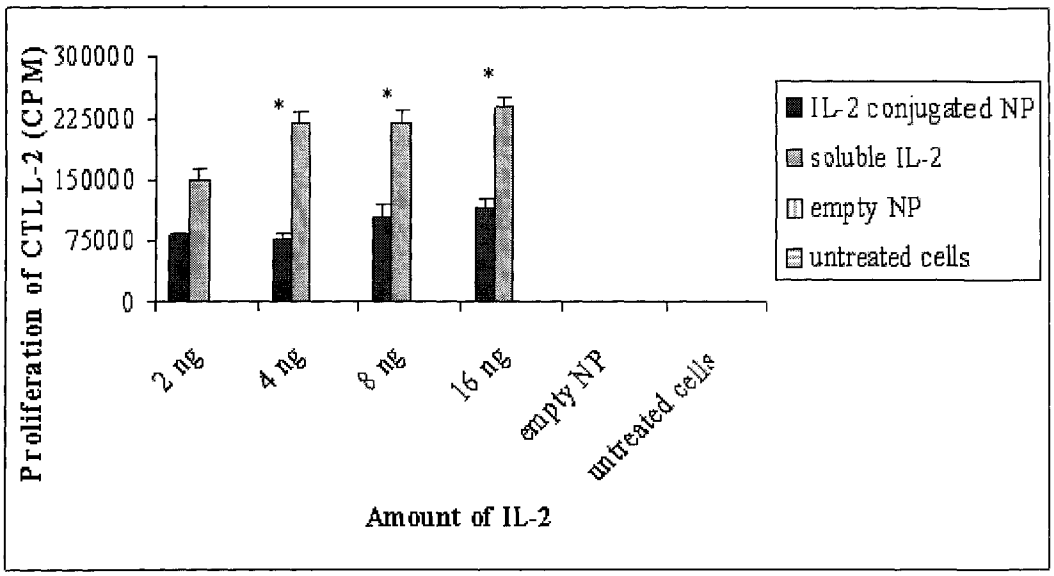


Figure 3.8 A. Comparison of bioactivity of IL-2 conjugated HSA nanoparticles with soluble IL-2, empty nanoparticles and untreated cells after 24 h culture. (mean ± S.D; n = 3). The amount of HSA nanoparticles was 500 μg in the samples. *p< 0.05

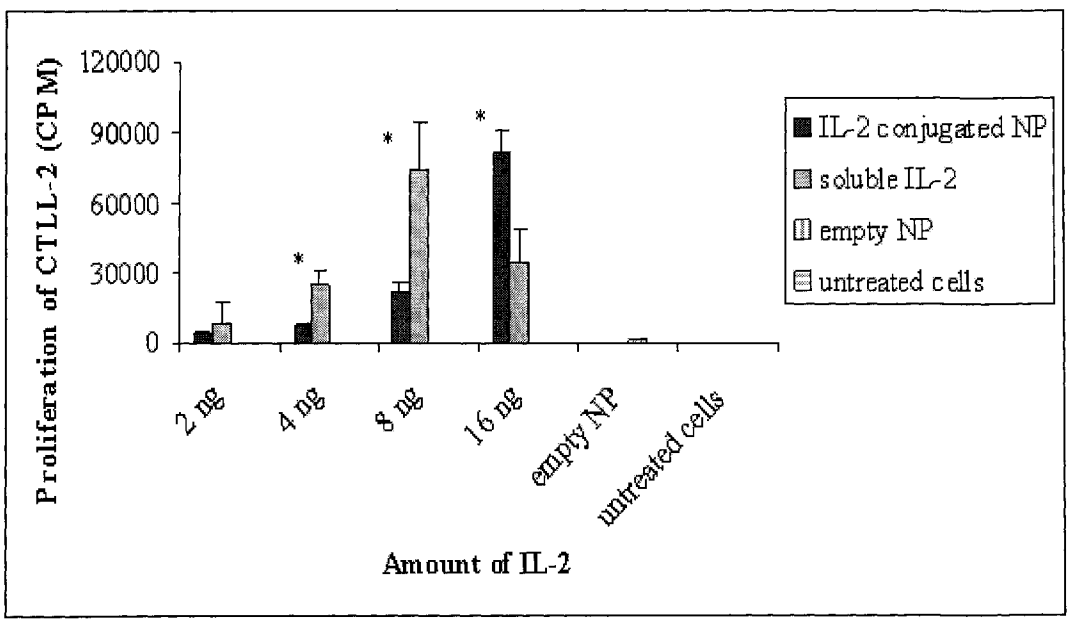


Figure 3.8 B Comparison of bioactivity of IL-2 conjugated HSA nanoparticles with soluble IL-2, empty nanoparticles and untreated cells after 4 day culture. (mean ± S.D; n = 3). The amount of HSA nanoparticles was 500 μg in the samples. *p< 0.05

Chapter 4

General discussion and conclusions

The main limitations of systemic drug delivery include an uneven bio-distribution throughout the body, the necessity of a large dose to achieve high local concentration and adverse side effects due to such high doses. There is now a growing understanding that innovative delivery systems would not only increase safety levels, but also improve the overall performance of drugs [235]. One of the primary objectives in the design of novel drug delivery systems is delivery of the drug to its site of action at a desired concentration for a desired time. This site-specific delivery will enhance efficacy of the drug and reduce unwanted side effects. Among the promising systems to achieve this goal are colloidal drug delivery systems [201].

Our study has investigated the development of a colloidal drug delivery system for interleukin-2, a clinically approved drug, which causes severe side effects in addition to its therapeutic effects. IL-2 can stimulate a variety of immunological mechanisms such as activation of NK cells, expansion of cancer antigen-specific T helper and cytotoxic T cells, and potentiation of their effector mechanisms. The therapeutic benefits of IL-2 are subject to its availability at the tumor site. However, the administration of high systemic doses of IL-2 causes various side effects. The objective of this study was to develop a delivery system that can reduce the systemic concentration of IL-2 but increase the accumulation of IL-2 at tumor sites and lymphoid organs. For this purpose, first, the encapsulation of IL-2 in MePEO-*b*-PDLLA block copolymer micelles was examined. The copolymer was synthesized and particles were characterized with respect to size, polydispersity and shape. However, no appreciable encapsulation

of IL-2 was observed in these particles. Next, the conjugation of IL-2 with HSA nanoparticles through disulfide linkage was assessed. To accomplish this, free thiol groups were introduced on the particles. The results demonstrate that IL-2 was successfully linked to HSA nanoparticles through disulfide bond formation. Moreover, IL-2 showed some degree of bioactivity after conjugation to particles.

Site-specific delivery of IL-2 at the tumor site and lymphoid organs using the particles will result in less systemic concentration and thereby fewer toxic effects. The lower amount of degradation of HSA-IL-2 particle will increase the half-life of IL-2. Future studies that involve testing of the formulation *in vivo* for characterizing pharmacokinetics, biodistribution, *in vivo* toxicity and antitumor efficacy of IL-2 nano-carriers are suggested.

The benefit of using HSA nanoparticles is its biocompatibility, easy storage, greater stability during storage and the possibilities for surface modification. Extreme care should be taken on the source of albumin. To reduce the chance of infectious diseases or other blood related disorders, blood donors for albumin production has to be carefully screened [194].

The novel formulation of IL-2 conjugated HSA nanoparticles are intended for intravenous administration. The leakiness of tumor vasculature enables the nanoparticles to enter the tumor. One obstacle against tumor targeted delivery by HSA nanoparticles is their non-specific uptake by the RES. HSA particles may rapidly be cleared and taken up from the circulation by RES (liver and spleen and macrophages). Absorption of PEO containing polymers on the HSA surface may be used in future studies as an additional strategy to prevent the uptake of HSA

particles by RES.

For delivery to the tumor-draining lymph nodes, the HSA nanoparticles can be administered subcutaneously near the tumor. The uptake of the particle into the lymphatics is a passive process, occurring naturally as the lymph is formed [236]. To allow significant accumulation in many lymph nodes, the particles can be injected subcutaneously at other sites as well. Another application would be the use of IL-2 nanoparticles to boost the immune system of HIV patients. The toxicity can be minimized by subcutaneous injection. For the management of pulmonary metastases and primary lung cancers, the nanoparticles can be administered by aerosol route. Furthermore, IL-2 nanoparticles would also find application in the treatment of autoimmune diseases. In this case intravenous administration of the particles can cause expansion of Treg cells, which regulate autoimmunity.

Alternative formulations that attracted attention for IL-2 delivery include PEG-IL-2 and liposomes. Since PEG-IL-2 only increased half-life but did not reduce toxicity, its clinical development has been abandoned [237]. Liposomes are promising because of their good biocompatibility and low toxicity. Liposomal IL-2 have been utilized to activate the immune system with decreased toxicity [238]. The delivery of IL-2 liposomes by inhalation was found to be well tolerated and feasible in patients with pulmonary metastases [239]. No significant toxicity was observed in this phase 1 trial. Nevertheless, further studies of inhalational IL-2 liposomes to determine its efficacy as an anti-cancer therapy is needed.

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