SYNTHESIS AND CHARACTERIZATION OF SPIONS-BROMELAIN-FOLIC ACID ON FOLIC ACID RECEPTOR POSITIVE CANCER MODEL

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Dedicated to

My ever-supportive Mama (Ashraf Razavi), Papa (Sohrab Nasiri), my sisters, my brothers and my colleague (Javad Hamzehalipour Almaki).

Specially dedicated to my beloved mother (Ashraf Razavi) and sisters (Setareh and Mahtab) who are everything in my life.

Thank you for being the best thing that ever happened in my life.

Love you all by very fabric of my being.

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ABSTRACT

Engineering of a physiologically compatible, stable and targetable delivery vehicle superparamagnetic iron oxide nanoparticles-Bromelain-folic acid (SPIONs-Br-FA) was reported. Initially, the synthesized bare SPIONs were coated with citric acid (CA) in order to increase biocompatibility, stability and solubility of the SPIONs. Moreover, through CA coating, carboxyl functional groups for further reactions were produced. Br (as an anti-cancer agent) and FA (as a targeting agent to the folic acid receptor positive (FAR+) cancer cells) were conjugated to the synthesized nanocarrier through 1-ethyl-3-(3-dimethylaminpropyl)carbodiimide hydrochloride/ N-hydroxysuccinimide (EDC/NHS) click chemistry. Subsequently, characterization and physico-chemical analyses were carried out through methods such as Fourier transform infrared spectroscopy, atomic absorption spectroscopy (AAS), dynamic light scattering, vibrating sample magnetometer, x-ray diffraction, transmission electron microscopy (TEM) and field emission scanning electron microscopy. The *in vitro* tetrazolium dye (MTT) assay and blood compatibility tests were performed to confirm the biocompatibility of the engineered nano delivery system. High level of SPIONs-FA binding to FAR+ cell lines (HeLa, MDA-MB-231 and 4 T1) compared to folic acid receptor negative (FAR-) cell lines (HSF 1184 and MDA-MB-468) was assured via qualitative and quantitative *in vitro* binding studies (Prussian blue assay and AAS analysis). The reason may be higher transport of SPIONs-FA through the mechanism of receptor endocytosis pathway into FAR+ cells in comparison with the mechanism of passive diffusion of SPIONs into the FAR- cells. Cytotoxicity studies carried out in human cell lines (HSF 1184, MDA-MB-468, MDA-MB-231 and HeLa) and mouse breast cancer cells (4 T1) showed significant dose advantage with SPIONs-Br-FA in reducing the half maximal inhibitory concentration (IC_{50}) values compared with neat Br. Through morphological observation studies by inverted microscope and acridine orange/ethidium bromide fluorescent staining method, it was disclosed that the cells had undergone apoptosis since the shrinkage as well as the apoptotic bodies were obviously seen. The results showed that SPIONs-Br-FA was a rewarding candidate to suppress the migration of the FAR+ cancer cells as well as to inhibit colony formation of the FAR+ cancer cells compared to neat Br. The percentage of apoptotic cells (apoptotic index) with more condensed and fragmented chromatin increased sharply in SPIONs-Br-FA treated cells compared to the neat Br. Overall, the SPIONs-Br-FA induced higher percentage of apoptotic cells than the neat Br. Moreover, after treatment protocol performance on 4 T1 tumor bearing mice, the qualitative and quantitative biodistribution study were carried out in vital organs and tumor using colorimetric method (AAS) and TEM method which indicate significant tumor targetability of SPIONs-FA. Finally, the tumor volume and inhibition growth rate were measured in 4 T1 tumor bearing mice treated with different SPIONs formulations to investigate the effectiveness of SPIONs-Br-FA. Administration of SPIONs-Br-FA through tail vein (three times a week) during the four-week treatment period reduced the tumor burden of tumor bearing mice and also increased their life-span when compared with SPIONs-Br and neat Br at same concentration of bromelain. In conclusion, the current results indicated the dual-functional synthesized SPIONs-Br-FA is a promising tool in the field of biomedicine, chiefly cancer therapy.

ABSTRAK

Kejuruteraan yang serasi secara fisiologi, stabil dan boleh menyasarkan sarana pemasukan partikel nano ferum oksida super paramagnet-*Bromelain*-asid folik (SPIONs-Br-FA) telah dilaporkan. SPIONs telah disaluti dengan asid sitrik (CA) untuk meningkatkan bioserasian, kestabilan dan keterlarutan SPIONs tersebut. Selain itu, melalui penyalutan CA, kumpulan-kumpulan karboksil berfungsi untuk tindak balas lanjutan telah dihasilkan. Br (sebagai agen anti-kanser) dan FA (sebagai agen penyasaran terhadap sel-sel kanser positif reseptor asid folik (FAR+)) telah dikonjugasi pada pembawa-nano yang disintesis melalui kimia klik 1-etil-3-(-dimetilaminopropil)karbodiimida hidroklorida/N-hidriksisusinimida (*EDC/NHS)*. Seterusnya, pencirian dan analisis kimia-fizik telah dijalankan melalui kaedah tertentu seperti spektroskopi inframerah transformasi Fourier, spektroskopi penyerapan atom (AAS), penyerakan cahaya dinamik, magnetometer sampel bergetar, pembelauan sinar-x, mikroskopi pancaran elektron (TEM) dan mikroskop elektron pengimbas pancaran medan. Asai *in vitro* pewarna tetrazolium (MTT) dan ujian serasian darah telah dijalankan untuk mengesahkan bioserasian sistem pemasukan nano yang dibina. Tahap pengikatan SPIONs-FA yang tinggi terhadap titisan-titisan sel FAR+ (He La, MDA-MB-231 dan 4 T1) berbanding dengan titisan-titisan sel negatif reseptor asid folik (FAR-) (HSF 1184 dan MDA-MB-468) telah dikenal pasti melalui kajian pengikatan *in vitro* kualitatif dan kuantitatif (asai biru Prusia dan analisis AAS). Ini mungkin disebabkan oleh pengangkutan yang lebih tinggi SPIONs-FA menerusi mekanisme tapak jalan endositosis reseptor ke dalam sel-sel FAR+ berbanding dengan mekanisme peresapan pasif SPIONs ke dalam sel-sel FAR-. Kajian kesitoksikan yang dijalankan pada titisan-titisan sel manusia (HSF 1184, MDA-MB-468, MDA-MB-231 dan HeLa) dan sel-sel kanser payudara mencit (4 T1) telah menunjukkan kelebihan dos yang signifikan dengan SPIONs-Br-FA dalam mengurangkan nilai-nilai separuh maksimum kepekatan yang melarang (IC_{50}) berbanding dengan Br tulen. Melalui kajian pemerhatian morfologi oleh mikroskop songsang dan kaedah pewarnaan pendarfluor akridina jingga/etidium bromida, di dapati bahawa sel-sel telah menjalani apoptosis kerana pengecutan sel dan jasad-jasad apoptosis jelas kelihatan. Hasil kajian menunjukkan bahawa SPIONs-Br-FA merupakan sel yang sesuai untuk menyekat migrasi sel-sel kanser FAR+ serta merencat pembentukan koloni sel-sel kanser FAR+ berbanding dengan Br tulen. Peratusan sel apoptosis (indeks apoptosis) dengan kromatin mampat dan tersepih meningkat secara mendadak dalam sel-sel terawat SPIONs-Br-FA berbanding dengan Br tulen. Secara keseluruhan, SPIONs-Br-FA mengaruh peratusan sel-sel apoptosis yang lebih tinggi berbanding dengan Br tulen. Malahan, selepas protokol rawatan pada mencit terkandung sel-sel kanser 4 T1, kajian bio-pengedaran kualitatif dan kuantitatif telah dijalankan pada organ-organ penting dan tumor menggunakan kaedah kolorimetri (AAS) dan TEM yang menunjukkan kebolehan penyasaran tumor yang signifikan oleh SPIONs-FA. Akhir sekali, isipadu tumor dan kadar perencatan tumbesaran diukur pada mencit terkandung sel-sel kanser 4 T1 setelah dirawat dengan pelbagai formulasi SPIONs untuk menyelidik keberkesanan SPIONs-Br-FA. Pemberian SPIONs-Br-FA melalui vena ekor (tiga kali seminggu) semasa tempoh rawatan empat minggu telah mengurangkan bebanan tumor pada mencit dan juga meningkatkan jangka hayat mereka semasa perbandingan SPIONs-Br dengan Br tulen pada kepekatan bromelain yang sama. Sebagai kesimpulan, hasil kajian semasa menunjukkan bahawa SPIONs-Br-FA berdwi-fungsi yang disintesiskan merupakan alat berpotensi dalam bidang bioperubatan, terutamanya terapi kanser.

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LIST OF ABBREVIATIONS

AAS	-	Atomic Absorption Spectroscopy
APTT	-	Activated partial thromboplastin time
AR	-	Androgen receptor
Br	-	Bromelain
CA	-	Trisodum citrate dihydrate
CI	-	Confidence interval
DLS	-	Dynamic Light Scattering
EGFR	-	Epidermal growth factor receptor
EPR	-	Enhanced permeability and retention
FA	-	Folic acid
FAR	-	Folic acid receptor
FAR-	-	Negative folic acid receptor
FAR+	-	Positive folic acid receptor
FB	-	Fibrin formation
FESEM	-	Field emission scanning electron microscopy
FRα (FAR)	-	Folic acid receptors
FT-IR	-	Fourier transform Infrared
FWHM	-	Full with at half maximum
HB	-	Hemoglobin
HCT	-	Hematocrit
HCT	-	Hard clotting time
HER2	-	Human epidermal growth factor receptor 2
HER3	-	Human epidermal growth factor receptor 3
HPLC	-	High performance liquid chromatography

ID	-	Injected dose
IGF-IR	-	Insulin-like growth factor receptor
MCHC	-	Mean corpuscular hemoglobin concentration
MCV	-	Average red blood cell size
MRI	-	Magnetic resonance imaging
MTT	-	Thiazolyl Blue Tetrazolium Bromide
NSCLC	-	Non-Small Cell Lung Cancer
PAMAM	-	Polyamidoamine
PARP	-	Poly(ADP-ribose) polymerase
PCV	-	Packed cell volume
PEG	-	Polyethylene glycol
PEI	-	Polyethylenimine
PRP	-	Platelets
PSMA	-	Prostate specific membrane antigen
PT	-	Prothrombin time
RBC	-	Red blood cells
RES	-	Reticuloendothelial system
Bare SPIONs	-	Superparamagnetic Iron oxide nanoparticles
SPIONs-Br	-	Bromelain conjugated citrate SPIONs
SPIONs-Br-FA	-	Bromelain and folate conjugated citrate SPIONs
SPIONs	-	Iron oxide nanoparticles coated with CA
SPIONs-FA	-	Folate conjugated citrate SPIONs
TEM	-	Transmission Electron Microscopy
TT	-	Thrombin time
VEGF-A	-	Vascular endothelial growth factor A
VEGFR	-	Vascular endothelial growth factor
VSM	-	Vibrating sample magnetometer
WBC	-	White blood cells
XRD	-	X-ray Diffraction

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CHAPTER 1

INTRODUCTION

1.1 Background of study

One of the most life threatening diseases is cancer where the number of new cases is growing increasingly (Boyle and Levin, 2008). According to the last report, breast cancer was the most common cancer in females and also the first most common cancer among population regardless of sex in Malaysia. There were 3,242 female breast cancer cases diagnosed and reported to NCR (National Cancer Registry) in 2007 which accounted for 18.1% of all cancer cases reported and 32.1% are all female cases. The age pattern in 2007 showed a peak ASR (age-standardised rate) at the 50-59 age groups. The incidence of breast cancer was highest among Chinese where the ASR was 38.1 per 100,000 population followed by Indian and Malay with the ASR of 33.7 per 100,000 population and 25.4 per 100,000 populations, respectively. The percentage of breast cancer detected at stage I and II was 58%. Cancer of the cervix was the third most common cancer among women and fifth most common cancer in the entire general population. There were a total of 847 cases diagnosed in 2007 registered at NCR. Cervical cancer incidence rate increased after 30 years old and peaks at ages 65-69 years. Compared among the major races, Indian women had the highest incidence for cervical cancer followed by Chinese and Malay. The ASR for Indian females was 10.3 per 100,000 populations. The percentage of breast cancer detected at stage I and II was 55% (Omar and Tamin, 2011). Figure 1.1 shows ten most frequent cancers in all residence and Figure 1.2 presents ten most frequent cancers among female in Malaysia in 2007.

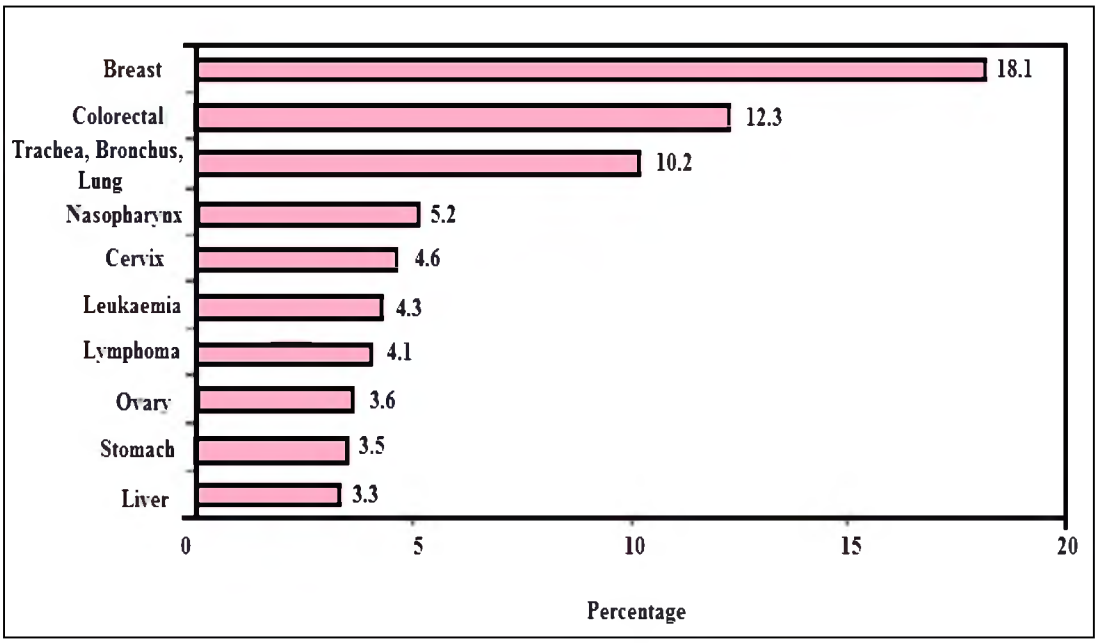

Figure 1.1 Ten most frequent cancers, all residence, Malaysia 2007 (Omar and Tamin, 2011).

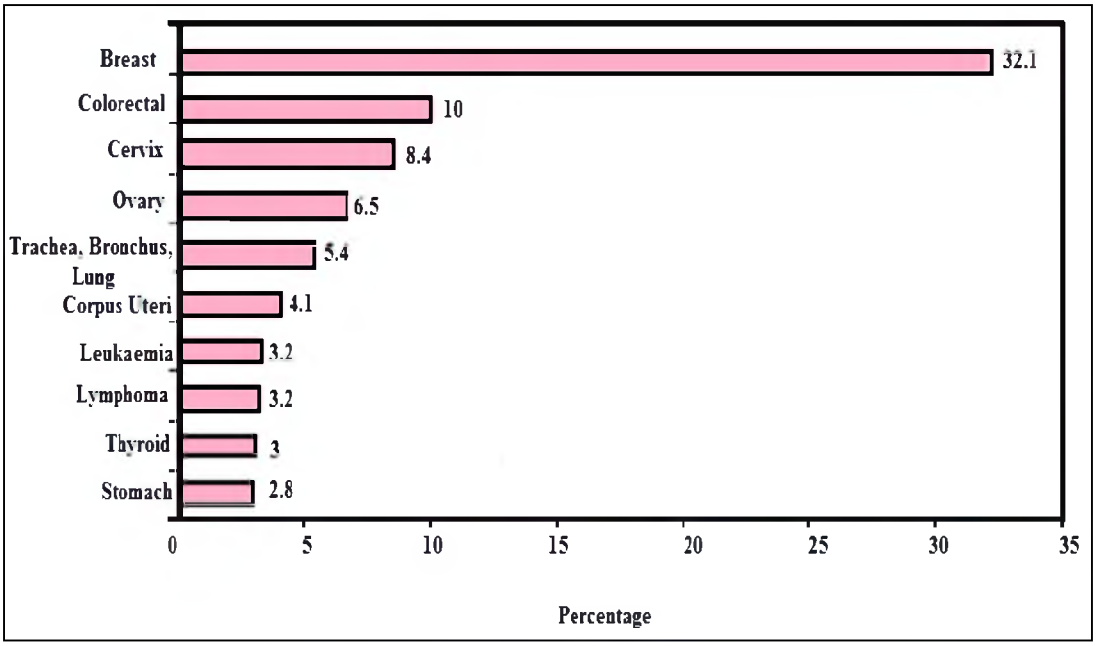

Figure 1.2 Ten most frequent cancers, female, Malaysia 2007 (Omar and Tamin, 2011).

In spite of the accelerated progress of diagnostics and treatments, substantial

improvements over the survival rate of patients have not yet been seen over the course of past few decades (Jemal *et al.*, 2010). Developing a novel approach to incur the detection of cancer in its early-stages as well as developing targetable therapies is a remarkable need.

Chemotherapy is the most practiced cancer treatment method in the world over the years. Nonspecific conventional chemotherapy normally leads to extreme side effects and is compromised because of its dose-limiting toxicity. Nanomaterials advances have made passive and active targeting strategies possible to boost up concentration of drugs inside tumor. Moreover, limiting the unwanted drug toxicity to healthy tissue is whereby achieved (Maeda, 2001; Allen, 2002; Torchilin, 2006). The targetable drug delivery is expected to eliminate troubles in conventional neat anticancer agents, including insolubility, accelerated clearance, unselective binding ability that leads to nonspecific toxicity towards healthy cells and decreases the drug dose delivered to the cancer cells (Ashley *et al.,* 2011). Since nano drug carriers offer longer half-lives in blood circulatory system compared to free drugs, they unfold a key potential to target the cancer cells. Increased amount of delivery to the cancer cells is highly dependent on the lowered total body clearance of the nano drug carriers. Additionally, due to the presence of poor lymphatic drainage and leaky blood vessels in the tumor site, retention and permeation of the nano drug carriers to the tumor site are highly enhanced. Since the conjugates find their way into the cancer cells through endocytosis, active drug molecules are released via either acid or intracellular enzymatic hydrolysis. Hence, drug internalization into the cancer cells is boosted up via raising the binding extent of conjugates to the cancer cells. This route, selective endocytosis, has been investigated by the attachment of targeting ligands to the nano carriers (Chau *et al.*, 2004).

Figure 1.3 illustrates the different localization of drug in targeted strategy by nano drugs compared with systemic treatment by classic drugs. For oral intake or intravenous injection of the classical drug, the bioactive component is distributed throughout the body without any distinctions between healthy and inflamed tissue. In targeting strategy, nano drugs are attached to the targeting agents whose ligands are overexpressed in interested areas. The nano drugs accumulate and the drug is

released in the specific area.

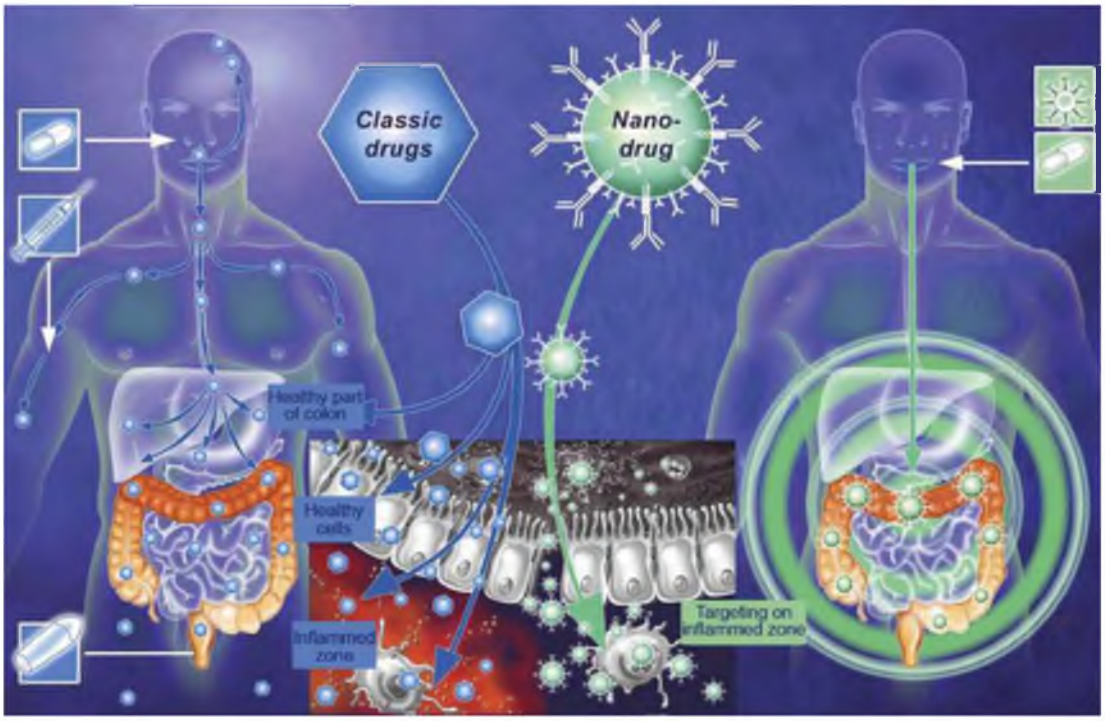

Figure 1.3 Nano drug vs. classic drug distribution in body (Laroui *et al.*, 2011).

Recent nanotechnological advances offer platforms to fabricate ultrasmall probes like superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs are well known for their invaluable function in biomedical applications like magnetic resonance imaging (MRI),intracellular magnetic hyperthermia, targeted drug delivery, cell tracking and labelling, localized therapy, etc. (Laurent *et al.*, 2008; Fang and Zhang, 2009). SPIONs are in preclinical studies as well as in early stage clinical trials (Laurent *et al.*, 2008; McCarthy and Weissleder, 2008). A variety of methods have been reported to synthesize SPIONs like micro emulsion, sono-chemical synthesis, thermal decomposition, hydrothermal synthesis and co-precipitation. (Woo *et al.*, 2004; Wu *et al.*, 2009). Co-precipitation is a neat and suitable method for synthesis of SPIONs smaller than 20 nm in diameter (Li *et al.*, 2013). Desired SPIONs for various biomedical applications are between 10 nm and 100 nm in diameter (Wahajuddin, 2012). Recent investigations have reflected the fact that SPIONs are highly favorable drug targeting platforms because of their rather poor toxic effects (Neuberger *et al.*, 2005) and high magnetic saturation magnitudes

(Bean and Livingston, 1959).

However, due to the hydrophobic nature of SPIONs, they are instable and prefer to aggregate in physiological condition (Jain *et al.*, 2005; Mahmoudi *et al.*, 2010). Moreover, the large surface area to volume ratio of SPIONs compels the tendency to aggregate thus limiting their naturally high level of surface energy (Vayssieres *et al.*, 1998). Therefore, organic or inorganic materials are used to coat the SPIONs surface to barricade agglomeration and ensure biocompatibility. Coating not only stabilizes the SPIONs, but also promotes the attachment of biological moieties to them; the particles are targeted to cells by attaching functional groups to the SPIONs. Citric acid ($C_6H_8O_7$) has been extensively used as a biocompatible and short-chained tri-carboxylic acid to stabilize SPIONs for different biomedical applications (Liu and Huang, 1999; Nigam *et al.*, 2011; Lapresta-Fernández *et al.*, 2011)

Since high level of targeting is not offered by SPIONs due to their physiochemical profiles, active biomolecules are attached to the surface of the SPIONs to heighten the targeting specificity of nanoparticles (Lee *et al.*, 2007; McCarthy and Weissleder, 2008; Goya *et al.*, 2008). Clinical utility of the SPIONs is significantly increased after being bonded to the contrast agents allowing the SPIONs to accumulate in the sites of interest (Artemov, 2003; Choi *et al.*, 2004; Leuschner *et al.*, 2006). Additionally, various studies have pointed out diverse approaches for active targeting of SPIONs by protein structures, nutrients and therapeutics. Internalization of structures attached to the SPIONs are inhibited due to the bulky and immunogenic nature of antibodies (Zhang *et al.*, 2002). Since nutrient pathways increase the uptake of SPIONs because of their direct linkage to cell proliferation process, most tumor types provide signals more excellently. Tumor cells are dependent to folic acid (FA) as it is one of the essential precursors in synthesis of DNA base (Weitman *et al.*, 1992; Garin-Chesa *et al.*, 1993; Ross *et al.*, 1994), , In normal cells, folate receptors are slightly expressed (Weitman *et al.*, 1992) and it assists the nanoparticles to conjugate with FA to be internalized to the cancer cells simultaneously expressing folate receptors (FAR+) through receptor-mediated endocytosis pathway due to high levels of penetration and affinity (Barz *et al.*, 2010).

In most of the studies (Müller *et al.*, 2008; Razjouyan *et al.*, 2015), mentioned, folic acid was used in combination with nanostructures other than citrate-coated maghemite. But, in this study, folic acid was conjugated to the SPIONs via the help of citric acid (CA) resulting in synthesis of a novel biomaterial with a monodisperse nature and desired characteristics offering targeting capabilities to track and attach to the FAR+ cancer cells while being highly blood compatible and remarkably reduced cytotoxicity.

Nowadays, the use of bromelain (Br) as an anticancer agent is fast becoming attractive. Several studies, both animal and human, indicate bromelain have antimetastatic activities (Pillai *et al.*, 2013). Bromelain due to its anti-inflammatory, mucolytic, antithrombotic, wound debridement and anticancer properties has undergone investigations as a cysteine proteinase extracted from pineapple (Ananascomosus). Bromelain also offers anti-tumorigenic properties so that it enhances chemotherapy effect in both *in vitro* and *in vivo* trials particularly in breast and pancreas cancers. Proteolytic component of Bromelain may be chiefly liable to its anti-tumor activity according to a recent review (Bala *et al.*, 2012). It is evident that glycosylated moieties providing cellular oncogenic survival pathways may be influenced since bromelain hydrolyses linkages of glycosides in glycoproteins. Moreover, there are merits to the disruption of the glycosidic linkages in the secreted mucin via proteolytic action of bromelain because it may disrupt the mucinous barrier and offers a more efficient passage for cytotoxic drugs (Pillai *et al.*, 2013).

In this research, to make the surface of the synthesized bare SPIONs (γ-Fe_2O_3) hydrophilic, functional groups for further surface functionalization were provided, nanoparticles agglomeration was prevented and absorption of CA onto the surface of nanoparticles was carried out leaving a carboxylic acid exposed on the surface. The final product was engineered by conjugation of bromalain (Br) and folic acid (FA) to the SPIONs (Citrate coated iron oxide nanoparticles). Briefly, in the study reported herein an attempt has been made to synthesize the SPIONs-Br-FA as a novel engineered delivery of bromelain to the FAR+ cancer cells.

1.2 Problem statement

Chemotherapy, mastectomy, and radiotherapy are conventional cancer treatment methods which are not completely successful and they induce many side effects on healthy tissues. Currently, immense numbers of different chemotherapeutic anticancer agents are available, but the problem is drugs that are more effective tend to be more toxic. For example, one of the most effective and widely used anticancer agent cisplatin, is reported to cause adverse effects including nausea, vomiting, diarrhea, hair loss, loss in ability to taste food, hiccups, dry mouth, dark urine, decreased sweating, dry skin, and other signs of dehydration which considerably limit its applicability (Santabarbara *et al.*, 2016). So, there is a need of a drug carrier system to minimize systemic side effects compared to chemotherapy by actively targeting the anticancer agent to the cancer cells.

Drugs used in classic chemotherapy are incapable of detecting cancer cells thus they influence both cancer cells as well as the healthy ones. Therefore patients tend to suffer from such classic conventional treatment. But, nano-drugs possess the capability to target the cancer cells actively since their surface can be functionalized via ligands that can specifically attach to the cancer cells.

On the other hand, metastasis treatment of tumors is unsuccessful by conventional methods. Metastasis is the secondary malignant growth at a distance from a primary site of cancer. Tumor itself can be treated by mastectomy or other treatment methods, but metastasis does not appear during the first stage of disease thus that it could not be detected and treated easily.

In the proposed study, folic acid will detect metastasis wherever it is and the complex will bind to the FAR+ cancer cell receptors to maximize the anticancer effect of bromelain on targeted cancer tissue (tumor site) and to minimize toxicity to the normal tissues (Wang *et al.*, 2011).

1.3 Research objectives

The objectives of study are as follows.

I. To synthesize and characterize SPIONs-Br-FA.
II. To evaluate *in vitro* and *in vivo* binding affinity of SPIONs-FA.
III. To investigate *in vitro* and *in vivo* cancer inhibition efficacy of SPIONs-Br-FA and to compare it with the efficacy of neat Br.

1.4 Scope of research

In order to achieve the objectives, the scope of the study was as follows:

i) The bare SPIONs (γFe_2O_3) were synthesized using co-precipitation method and coated with citric acid (CA). The prepared bare SPIONs and coated SPIONs were then analyzed using FT-IR. In addition, iron oxide concentration was determined by atomic absorption spectroscopy (AAS).

ii) Then, bromelain (Br) as an anticancer agent and folic acid (FA) as a targeting agent were conjugated using 1-ethy-3-(3-dimethylaminopropyl) cabodiimide (EDC)/N-hydroxysuccinimide (NHS) click chemistry method to the coated SPIONs. The loading efficiencies of bromelain and folic acid were determined using Bradford assay and HPLC, respectively.

iii) The functionalized SPIONs were characterized by FT-IR, AAS, VSM, DLS, XRD, FESEM and TEM equipment.

iv) The biocompatibility of synthesized delivery system in each synthesis step was determined using MTT, haemolysis, blood aggregation and blood clotting time assays.

v) Binding ability of the developed coated SPIONs and SPIONs-FA to HSF 1184, MDA-MB-231, MDA-MB-468, HeLa and 4 T1 cells was investigated using qualitative (Prussian Blue Assay) and quantitative (AAS) methods.

vi) The MTT assay was carried out on HSF 1184, MDA-MB-231, MDA-MB-468, HeLa and 4 T1 cells to find the cytotoxicity effect of synthesized formulation in each step.

vii) The biodistribution study was carried out in all important organs using colorimetric (AAS) and TEM methods in established 4 T1 tumor bearing mice model.

viii) In the last step, after treatment protocol performance on 4 T1 tumor bearing mice model, the tumor volume and survival rate was measured to investigate the effectiveness of the proposed formulation on tumor bearing mice.

In this study, HeLa, 4 T1 and MDA-MB-231 cancer cell lines were used as (FAR+) targeted cells while HSF 1184 and MDA-MB-468 was used as (FAR-) non-targeted cells.

1.5 Significance of study

The synthesized SPIONs-Br-FA is a novel and safe delivery system to

minimize the side effect of anticancer agents to the normal tissues and to maximize their toxic effect on tumor site. In this study, a novel nano delivery system (SPIONs-Br-FA) was developed for the first time based on our hypothesis that the effectiveness of SPIONs-Br-FA on cancer cell was improved compared to neat Br treatment. Targeted delivery of bromelain as an anticancer agent to the cancer cells leads to the possibility of metastasis treatment and minor systemic side effects compared to the neat Br treatment. Another significance of this study includes the enhancement of bromelain delivery in the non-invasive and cost-effective manner utilizing minimal dosage of drug to achieve maximum potency.

1.6 Thesis Organization

The thesis is divided into five chapters. The first chapter describes the research background, problem statement, research objectives, scope of research and significance of study.

The second chapter consists of comprehensive literature review based on the research topics. In this chapter, the description on the related literatures such as barrier to conventional cancer treatments, tumor vasculature, drug delivery, nanotechnology advances in drug delivery, concept of passive and active targeting, active targeting by folic acid and recent work on bromelain are reviewed.

The third chapter explains the methodology used in this study. Initially, it describes the details and procedures to synthesize and characterize SPIONs-Br-FA and finally explains *in vitro* and *in vivo* assessments of synthesized formulations. This chapter also includes the list of materials used in this research.

Forth chapter exhibits and discusses the results obtained. This chapter has three main sections: (i) development and characterization of SPIONs-Br-FA, (ii) *in vitro* tests including biocompatibility studies, binding studies, cytotoxicity studies, morphological studies of cells, scratch motility and clonogenic assays, and (iii) *in*

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