

Tumor microenvironment and nanotherapeutics

Meenakshi Upreti, Amar Jyoti*, Pallavi Sethi*

Department of Pharmaceutical Sciences and Cancer Nanotechnology Training Center, College of Pharmacy, University of Kentucky, Lexington, KY, USA

*These authors have contributed equally to this work.

Corresponding to: Meenakshi Upreti, PhD. 555, College of Pharmacy, University of Kentucky, Lexington, KY, 40536, USA. Email: m.upreti@uky.edu.

Abstract: Recent studies delineate a predominant role for the tumor microenvironment in tumor growth and progression. Improved knowledge of cancer biology and investigation of the complex functional interrelation between the cellular and noncellular compartments of the tumor microenvironment have provided an ideal platform for the evolution of novel cancer nanotherapies. In addition, multifunctional “smart” nanoparticles carrying imaging agents and delivering multiple drugs targeted preferentially to the tumor/tumor microenvironment will lead to early diagnosis and better treatment for patients with cancer. The emerging knowledge of the tumor microenvironment has enabled rational designing of nanoparticles for combinatorial treatment strategies that include radiotherapy, antiangiogenesis and chemotherapy. This multimodality approach is thus expected to achieve therapeutic efficacy and enhance the quality of life of cancer patients. This review highlights the unique characteristics of the tumor microenvironment that are exploited by nanotechnology to develop novel drug delivery systems aimed to target the tumor/tumor microenvironment.

Key Words: Tumor microenvironment; nanoparticles; endothelial cells; enhanced permeability and retention (EPR) effect; multiple drug resistance (MDR)



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Introduction

The grand simplification of cancer in research until the end of twentieth century was that it is a disease of the cells. Therefore implying that the disease may be better understood by identifying the genetic changes resulting in altered proteins that disrupt the cell's communication network causing signals to be garbled, amplified or misdirected, hijacking what was once the normal communication to achieve uncontrolled growth of these genetically altered cells. However, the recent avalanche of information reveals that cancer is actually a dynamic milieu of neoplastic cells and a complex array of non-neoplastic cells that are recruited from the neighboring local or distant host tissue to the tumor microenvironment establishing a favorable niche for the growth of complex tissues that we call tumors (*Figure 1*) (1-3). These non-neoplastic cells that constitute the tumor microenvironment facilitate tumor

development by providing extracellular matrices, cytokines, growth factors, mechanical cues, and vascular networks for nutrient and waste exchange (4). More than 80% of the tumor burden is contributed by derivatives of epithelial tissues, called Carcinomas where the non-neoplastic tumor microenvironment accounts for 30-99% of the tumor mass. *Figure 2* elucidates the localization of different cell types that exist in the tumor stroma of histological specimens of Cholangiocarcinoma (5). Thus in the clinical setting it becomes mandatory to understand mechanisms to block the complex crosstalk between cancer cells, their non-neoplastic host cells and the surrounding extracellular matrix that constitute their local environment.

The significant abnormalities in the tumor microenvironment and its cells, such as an acidic pH, altered redox potential, up-regulated proteins and hyperthermia have led to the idea of using stimulus-responsive nanopreparations in antitumor applications (6). This approach

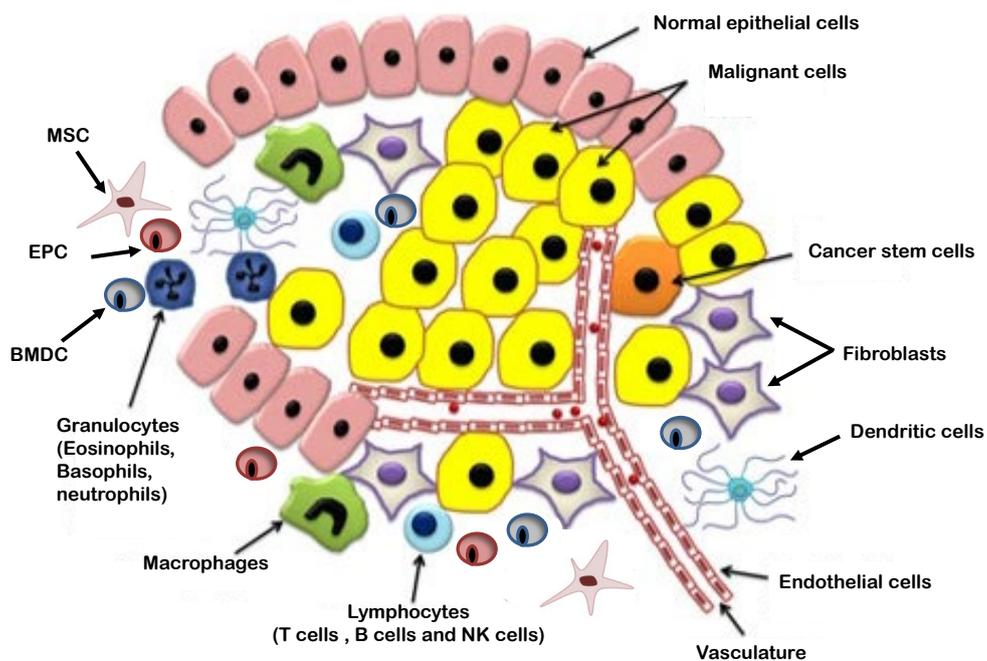


Figure 1 The primary tumor microenvironment. The primary tumor microenvironment consists of tumor cells surrounded by normal epithelial cells, mesenchymal stem cells (MSC), endothelial progenitor cells (EPCs) and various bone marrow derived cells (BMDC). Presence of heterogeneous cells and their secreted soluble factors, signaling molecules, extracellular matrix and mechanical cues within the tumor microenvironment promote neoplastic transformations, support tumor growth and invasion (Modified from www.Cernostics.com)

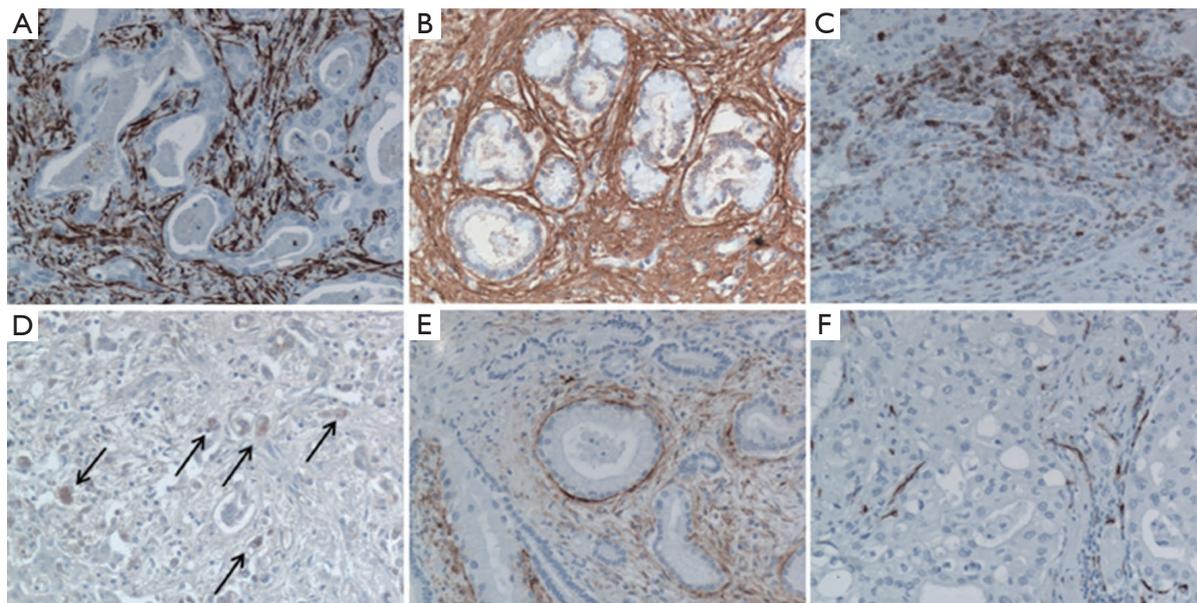


Figure 2 Phenotyping the tumor reactive stroma in Cholangiocarcinoma (CCA). Immunohistochemistry of different markers to characterize cells and structural components of the tumor reactive stroma in CCA: A. cancer-associated fibroblasts [CAFs] (α -SMA); B. extracellular matrix [ECM] (fibronectin); C. inflammatory cells (CD45); D. tumor-associated macrophages [TAM] (CD206, arrows); E. lymphatic endothelial cells (Podoplanin); F. vascular endothelial cells (CD34). Histological specimens were derived from surgical liver resection of patients with Intrahepatic cholangiocarcinoma (iCCA). Original magnification: 200 \times , adapted from (5)

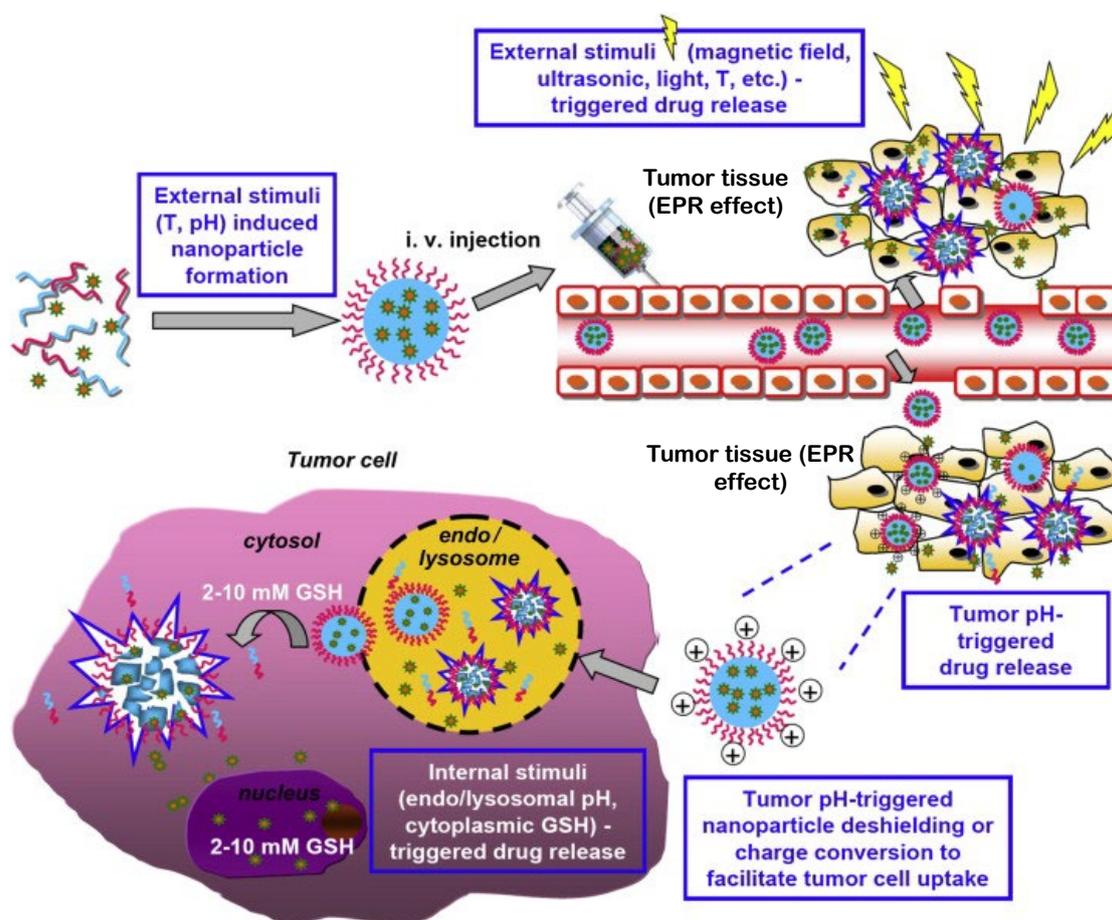


Figure 3 Stimuli responsive nanopreparations as emerging drug delivery and controlled drug release systems. The various stimuli are applied as following: (I) External stimulus such as temperature (T) and pH is utilized to facilitate formation of nanoparticles; (II) External stimuli such as magnetic field, ultrasonic, light, and temperature allows for remotely controlling the precision of spatial and temporal drug release; (III) acidic tumor pH (6.5-7.2) is utilized to trigger drug release and/or reverse shielding of nanoparticles at tumor site thereby enhancing tumor cell uptake of nanoparticulate drugs; and (IV) intracellular environments such as low pH in endo/lysosomal compartments and high redox potential in cytoplasm are utilized to improve intracellular drug release inside tumor cells adapted from (8)

is being further extended to design nanopreparations that respond to external stimuli like magnetic field, light and ultrasound for controlled drug release, improved drug internalization and regulation of the intracellular drug fate, resulting in an enhanced tumor targeting and antitumor effect *Figure 3* (6-8). Nanotechnology has thus become the emerging field of stimulus-responsive nanoformulations termed “smart drugs” in cancer which (I) utilizes the altered tumor environment to facilitate accumulation of the systemically delivered chemotherapy at the tumor site and (II) enables specific targeting of the tumor and/or tumor microenvironment to achieve tumor growth inhibition (9) and enhanced therapeutic efficacy (10).

Vascular pathophysiology and EPR effect in cancer nanotherapeutics

One of the six hallmarks that have been proposed during the development of cancer is sustained angiogenesis (11,12) where the tumors develop their own neovasculature from the existing host microenvironment for nourishment (13). These blood vessels produced within tumors by chronically activated angiogenesis and an unbalanced mix of proangiogenic signals are typically aberrant (14,15). These structural abnormalities result in a leaky vasculature and a poor lymphatic drainage system (16) which causes a differential interstitial pressure (17). The 10-100 nm

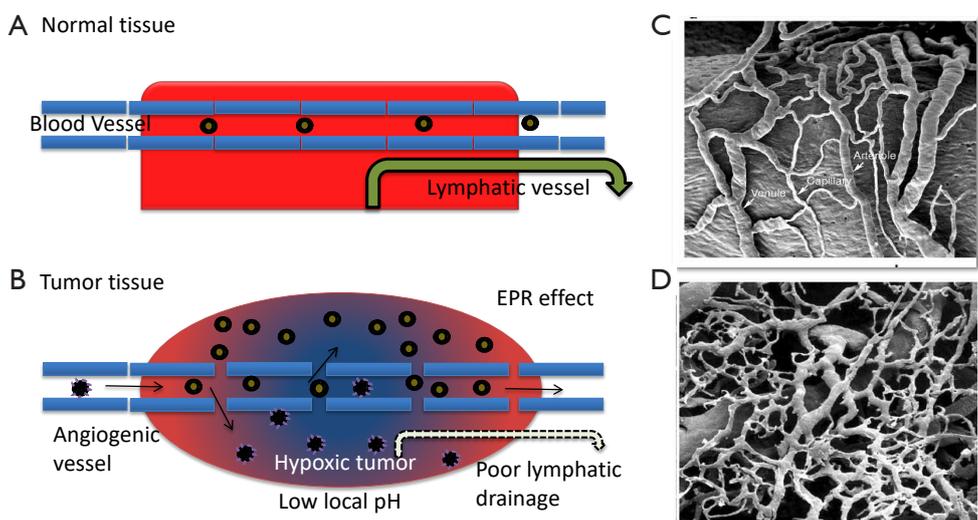


Figure 4 Vascular pathophysiology and EPR effect in nanoparticle delivery. Scheme representing the microvasculature of normal (A) and tumor (B) tissue. Poorly developed leaky vasculature allows 10-100 nm sized nanoparticles to extravasate and gets accumulated with in solid tumor. Within tumor depending on their sustained drug release properties, nanoparticles keep releasing active drug for significantly longer time point. Nanoparticles cannot leak through the intact blood vessels, so it considerably decreases the systemic toxicity. Scanning electron microscopic (SEM) imaging showing simple, organized arrangement of arterioles, capillaries, and venules in normal rat carotid sinus (C), on the contrary xenograft of human tumor in nude mice depicts abundant microvasculature lacking the hierarchy of blood vessels (D) SEM adapted from (21,22)

nanoparticles serving as delivery systems for drugs and small molecules like DNA/RNA utilize this difference in pressure to preferentially accumulate and be retained in tumors unlike the free drugs or small molecules that rapidly undergo renal filtration (18,19). This phenomenon of enhanced permeability and retention (EPR) effect has shown that the retention time of drugs packed in nanoparticles is ten times higher than that of free drugs at the tumor site (20). Hence, this EPR effect attributed to the leaky tumor vasculature is considered as a boon for drug-delivery systems within the nanosize range as described in *Figure 4*.

Role of altered pH dynamics in the tumor microenvironment in nanotechnology

Tumors contain oxygenated and hypoxic regions (23) and therefore unlike normal cells that derive the bulk of their ATP through mitochondrial oxidative phosphorylation, most cancer cells by what is referred to as 'Warburg effect' transition to the less efficient method of glycolysis for energy production, releasing as large amount of lactic acid (24). This method for energy production provides several advantages to the tumor including adaptation to a low oxygen environment and the acidification of the surrounding microenvironment, which

promotes tumor invasion and suppresses immune surveillance (25). Nanotechnology utilizes this phenomenon to design pH-sensitive nanoparticles that are stable at a physiologic pH of 7.4, but degraded to release active drug in target tissues in which the pH is less than physiologic values, such as in the acidic environment (6.7-6.9) of tumor cells (26-29). Currently, nearly all successful cancer chemotherapy regimens use a paradigm of multiple drugs given simultaneously. This type of multicomponent chemotherapy has been first demonstrated in nanoscale delivery vehicles by the O'Halloran group where two cytotoxic agents are co-encapsulated into 100 nm liposomes that are stable in serum but release their drug in the low-pH endosome, potentially leading to synergistic drug activities (30). This system is continuously being improvised to encapsulate new drug combinations and covalently attached targeting ligands to direct drugs specifically to the tumor site. Recently it has also been shown that nanoparticles composed of weak polybases when exposed to a pH gradient tend to accumulate preferentially and increase in size/swell when in the low pH regions by a phenomenon termed "pH phoresis". The tumor tissues provide the required low pH microenvironment where the polybase nanoparticles upon accumulation increase in size and get caught in the fenestrated tumor vasculature, facilitating enhanced delivery of drugs to the tumor site (31).

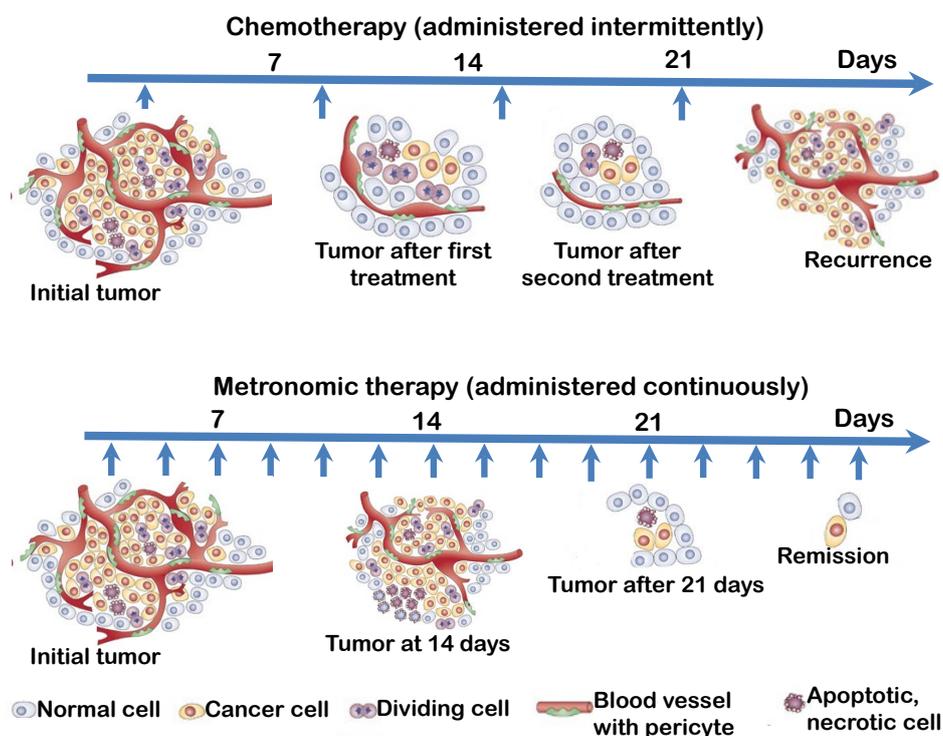


Figure 5 A comparison of the effect of Chemotherapy and metronomic therapy in cancer. A schematic representation elucidating the importance of metronomic dosing over traditional maximum tolerated dosing (MTD) in intermittent chemotherapy. Traditional chemotherapeutic regimen is often associated with systemic toxicity and recurrence of tumor after several days of treatment. However, in metronomic therapy, fractionated MTD for a period of time, is less toxic and is effective in tumor growth inhibition and results in remission of the disease

Controlled release in the tumor microenvironment by nanocarriers simulates metronomic therapy

Traditional chemotherapeutic regimens incorporate the “maximum tolerated dose” in the treatment protocols as a standard of care (32). This results in a concomitant overt systemic toxicity which has made it mandatory for the imposition of rest periods between cycles of therapy—a practice that not only involves re-growth of tumor cells, but also growth of selected clones resistant to the therapy reverting to the growth of more malignant metastatic tumors with no therapeutic response. A new philosophy expected to overcome the problems encountered by the conventional treatment regimens that has been introduced by Judah Folkman and Robert Kerbel (33,34) and termed ‘metronomic therapy’ by Douglas Hanahan (35,36) involves a schedule which consists of low doses of chemotherapeutic drugs administered without extended rest periods (Figure 5). The novelty in this concept is in the targeting of

the tumor microenvironment, particularly the endothelial cells which are more sensitive to the consistent low dose drug administration than tumor cells, inhibiting tumor angiogenesis eventually resulting in tumor growth inhibition (37). Upon literature survey we found the endothelial cell types to be more sensitive than the tumor cell types to the anticancer drug, Topotecan. Metronomic dosing was more effective in killing the endothelial cells in comparison to the tumor cell types (Table 1). Interestingly, this sustained delivery and controlled release preferentially at the tumor/tumor microenvironment site is a challenge which drives the design of various drug delivery strategies that strive to revolutionize the way drugs exert their actions. Nanosized drug carriers due to their small size, relatively high surface area, influence on biodistribution, their stabilizing effect on therapeutic agents and their ability to make drugs available for intravascular delivery at the tumor site facilitate sustained release of active drug over a period of time simulating the action of metronomic therapy in cancer (44).

Table 1 Response of human tumor and endothelial cells to Topotecan administered as conventional chemotherapy and low dose metronomic therapy. A literature survey representing the Topotecan (TPT) IC50 values for most commonly used tumor and endothelial cell types revealed that endothelial cell types are more sensitive to TPT concentrations compared to different tumor cell type

Cell type	Cell line	IC50 (μM)	References
Breast cancer	MCF-7	0.218	(38)
Prostate cancer	PC-3	0.0935 \pm 0.028	(39)
Non small cell lung cancer	NCI-H460	0.598 \pm 0.025	(40)
Glioma	U251	1.2	(41)
Liver tumor	MRP4/HepG2	1.159 \pm 0.168	(42)
Ovarian cancer	HeyA8	0.025* (MD =0.024)	(43)
Primary endothelial cells	HUVEC	0.012* (MD =0.001)	(43)
Endothelial cells	EA.hy926	0.13	(41)

*In support of metronomic therapy, Merritt and coworkers [2009] have reported that while metronomic dosing (MD) doesn't affect the tumor cell lines, it increases the sensitivity of in Human Umbilical Vein Endothelial Cells (HUVECs) to TPT

Tumor microenvironment and prodrug therapy

Prodrugs are derivatives of drug molecules that can undergo a transformation by an enzyme, chemical or environmental stimuli to release the active parent drug *in vivo* (45). A drug which is highly cytotoxic or has a short half-life in circulation may now be administered in an inactive state as a nanoformulation or “prodrug” targeted to the tumor/tumor microenvironment via tumor specific molecules. Upon reaching its destination, the tumor environment facilitates its’ conversion to an active form. This tumor-activated prodrug therapy functions by attacking both the tumor and stroma cells through a “bystander effect” without selectively deleting the target-producing cells, therefore further minimizing resistance and toxicity. Matrix metalloproteinase-2 (*MMP-2*) is a *stroma*-derived *MMP* belonging to the type IV collagenase family playing a critical role in the degradation of basement membranes and the extracellular matrix. The overexpression of matrix metalloproteinase-2 in melanoma has been shown in a number of preclinical as well as clinical investigations. A water-soluble maleimide derivative of doxorubicin, incorporating a matrix metalloproteinase-2-specific peptide sequence developed by Mansour *et al.* has been shown to have high affinity for the cysteine-34 position of circulating albumin (46). The albumin-bound form of the polymer-drug conjugate was efficiently cleaved by the matrix metalloproteinase-2 enriched in the tumor stroma liberating free doxorubicin. The tumor microenvironment pH and redox potential were other stimuli that triggered drug release triggers at the tumor site (47). Cisplatin, an antiproliferative agent being used

in the treatment of cancer since the 1970's is known for its' severe side effects that include nephrotoxicity, neurotoxicity (ototoxic), and emetogenic (nausea and vomiting) has been shown by researchers from Lippard's and Farokhzad's group at MIT and Harvard respectively for safer and more effective prostate cancer therapy *in vivo* by the targeted delivery of a cisplatin prodrug. Being highly hydrophilic (water soluble) the half-life of cisplatin is 43 minutes with approximately 1/4th being eliminated within the first 24 hours (90% renal clearance). Encapsulation of the hydrophilic drug in a hydrophobic nanoparticle not only makes it an inactive prodrug but increases its half-life in circulation by 5 times and when coated with prostate specific membrane antigen (PSMA) facilitates targeted delivery of cisplatin to prostate cancer cells (48).

Preferential targeting of nanoparticles helps overcome multiple drug resistance (MDR) in cancer

MDR continues to remain a major unresolved challenge in clinical cancer chemotherapy (49). In the clinic, multidrug resistance occurs in over 50% of patients, whose cancer relapses, accounting in large part for the high mortality associated with cancer. Solid tumors exist in an intimate relationship with the surrounding microenvironment, and it is the dynamics of this heterogeneous and ever changing ecosystem that contributes to the initiation and progression of the disease (50-54). In addition to initiating and supporting the tumorigenic process, a permissive microenvironment can also affect the sensitivity of tumor

cells to drug treatment (55). The three-dimensional structure of the tumor tissue and the composition and organization of the extracellular matrix (ECM) and stromal components contribute to marked gradients in drug concentration, increased interstitial fluid pressure and metabolic changes, all of which may alter the resistance of tumor cells to cytotoxic agents and radiation (56-61). The tumor microenvironment/architecture has been shown to significantly contribute to the emergence of therapeutic resistance and thus the need for targeting and manipulating this complex symbiotic interplay to overcome MDR (62). The tumor microenvironment induced multidrug resistance occurs via (I) cell-cell and cell-ECM adhesion; (II) cell communication; (III) alterations in mechanosensing; (IV) Phenotypic transitions; and (V) protective quiescence (63). One of the most common mechanisms that has been shown to confer simultaneous resistance to different drugs relies on drug efflux from cancer cells mediated by ATP-binding cassette (ABC) transporters (64). A novel mechanism for the acquisition of drug resistance by tumor endothelial cells (TECs) in a tumour microenvironment to paclitaxel through greater mRNA expression of multidrug resistance 1, which encodes P-glycoprotein, as compared with normal endothelial cells has also been reported. High levels of vascular endothelial growth factor in tumour-conditioned medium were found to be responsible for the upregulated P-glycoprotein expression (65). Nanoparticles with affinity for specific receptors (66) in the tumor/tumor microenvironment when entering the cells, are usually enveloped by endosomes via receptor-mediated endocytosis, thereby bypassing the recognition of P-glycoprotein, one of the prominent ABC transporters mediating multidrug resistance, resulting in the increased intracellular concentration of drugs (67). Human serum albumin encapsulated paclitaxel (also known as Abraxane) is a clinically successful candidate that has been used to target the microenvironment utilizing the high affinity of a 60-kDa glycoprotein, gp60 located on the surface of endothelial cells displays for the albumin-paclitaxel complex (68,69). The albumin-paclitaxel complex when released into the subendothelial space is further enriched by another glycoprotein named SPARC (secreted protein, acidic and rich in cysteine) that binds to albumin with high affinity and has a significant homology to gp60 (70). We have identified Galectin-1, as a tumor vasculature associated protein (71) that is further specifically upregulated in endothelial cells in response to radiation exposure (72). It also serves as a major receptor for the

33 a.a. antiangiogenic peptide Anginex (73) and is thus a promising candidate for radiation enhanced delivery of chemotherapy via Anginex conjugated drug loaded nanoparticles. This multifunctional approach utilizing three modalities viz.: radiation, antiangiogenesis (anginex) and nanosized chemotherapy that is being developed in our laboratory to preferentially target the solid tumor is expected to provide a safer and more effective cancer chemoradiation therapeutic application (72). Multifunctional nanoparticle formulations designed to allow the drug to bypass the efflux of pump transporters or combination delivery and drug efflux modulation simultaneously (74) are now being actively investigated facilitating personalized and tailored cancer treatment (75). These multifunctional nanoparticles are also designed with additional capabilities like targeting ligand and image contrast enhancement that allow the nanoparticle to be used for theranostic imaging where therapy is combined with diagnosis, particularly suitable for disease as complex as cancer. The $\alpha_v\beta_3$ -integrin receptor is predominantly used for targeting vascular endothelial cells, as it is elevated in these cells during angiogenesis. Imaging agents targeting $\alpha_v\beta_3$ have been developed for MRI (76-79), PET (80) and fluorescence imaging (80-84). A tumor-homing peptide CREKA (Cys-Arg-Glu-Lys-Ala) that forms a distinct meshwork specifically in the tumor stroma synthesized by Simberg and colleagues, has been shown to facilitate accumulation of a CREKA-conjugated superparamagnetic iron oxide (SPIO) nanoparticles in both tumor vessels and stroma, resulting in intravascular clotting in tumor blood vessels. This intravascular clotting further attracts more nanoparticles into the tumor, amplifying the targeting. Such multifunctional targeted-SPIO nanoparticles allow for, (I) high specificity for tumor homing; (II) enhanced magnetic resonance imaging (MRI) in tumor; (III) physical blockade of tumor vessels by local embolism. The clotting caused by CREKA-SPIO nanoparticles in tumor vessels is expected to also improve tumor detection by optical imaging techniques (85). *Figure 6* shows a scheme for the design of multifunctional nanoparticles.

Conclusions and perspective

The emerging body of literature reveals that tumors are not merely collections of disorganized tumor cells but maladjusted living entities composed of neoplastic cells and surrounding non-neoplastic cells, termed the tumor microenvironment that are recruited by their neoplastic neighbors to provide essential support for the progressive

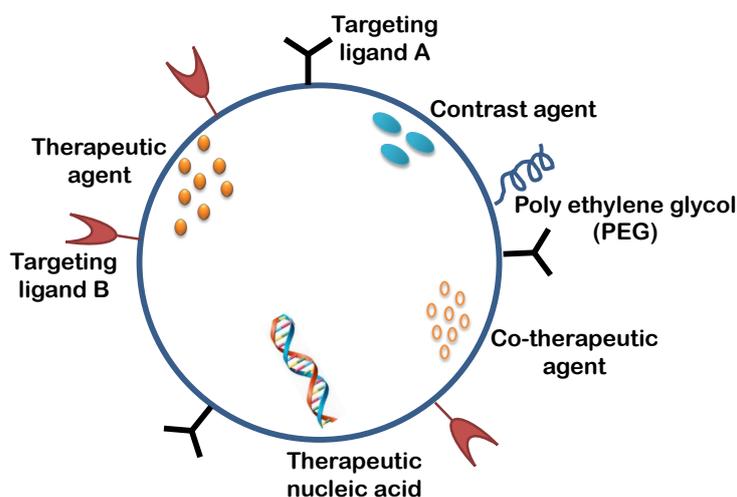


Figure 6 Scheme of Multifunctional nanoparticles used in cancer nanotherapeutics. The multifunctional nanocarriers are able to carry multiple therapeutic agents that may have a biological or non-biological origin. Impregnation of the nanocarrier with poly ethylene glycol termed PEGylation prolongs circulation time and reduces immunogenicity of nanoparticles, and impregnation of contrast agent/imaging agent makes this nanoparticulate formulation more traceable with in solid tumors. Conjugating targeting agents like antibodies and ligands is an approach to actively target tumor and endothelial cells within tumor microenvironment with minimum toxicity to other cell types. Hence, advances in the design of multifunctional nanoparticles is focused on developing the ideal nanocarrier that can effectively carry optimal drug to solid tumor, bring about tumor growth inhibition and prevent its progression

parasitic growth of the neoplasm. There is compelling evidence to indicate appearance of major structural and functional changes at the interface between tumor cells and adjacent host cells in the cancer microenvironment during the growth and progression of the neoplasm. A better understanding of this intricate ecosystem comprising the complex nature of tumor cell, host cell interactions, as well as cell-ECM interactions inside a tumor, has led to improved cancer therapies. The emerging field of cancer nanotechnology exploits these unique characteristics of the tumor microenvironment and tumor angiogenesis to design new drug delivery systems that specifically target anti-cancer drugs to tumors. National Cancer Institute has taken recent initiatives to harness the power of nanotechnology to radically change the way cancer is currently being diagnosed, imaged and treated. The nanotechnology market is expected to be worth \$1 trillion by 2015 as predicted by the US National Science Foundation. A combination of classical chemo and radiotherapy with anti-inflammatory and antiangiogenic strategies targeting the tumor microenvironment is required to reach long-term efficiency. With the growing number of clinical trials of nanotherapies associated with different targeting strategies and combined with radiotherapy or with conventional chemotherapy, provide adequate evidence of the success of these therapies in the future (86). Nanocarriers,

particularly multifunctional systems are thus expected to exist as the main therapeutic arsenal in the near future and play a major role in changing the very foundations of cancer diagnosis, treatment and prevention.

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