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Therapeutic application of anti-angiogenic nanomaterials in cancers†

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Angiogenesis, the formation of new blood vessels from pre-existing vasculature, plays a vital role in physiological and pathological processes (embryonic development, wound healing, tumor growth and metastasis). The overall balance of angiogenesis inside the human body is maintained by pro- and anti-angiogenic signals. The processes by which drugs inhibit angiogenesis as well as tumor growth are called the anti-angiogenesis technique, a most promising cancer treatment strategy. Over the last couple of decades, scientists have been developing angiogenesis inhibitors for the treatment of cancers. However, conventional anti-angiogenic therapy has several limitations including drug resistance that can create problems for a successful therapeutic strategy. Therefore, a new comprehensive treatment strategy using antiangiogenic agents for the treatment of cancer is urgently needed. Recently researchers have been developing and designing several nanoparticles that show anti-angiogenic properties. These nanomedicines could be useful as an alternative strategy for the treatment of various cancers using anti-angiogenic therapy. In this review article, we critically focus on the potential application of anti-angiogenic nanomaterial and nanoparticle based drug/siRNA/peptide delivery systems in cancer therapeutics. We also discuss the basic and clinical perspectives of anti-angiogenesis therapy, highlighting its importance in tumor angiogenesis, current status and future prospects and challenges.

1. Background: cancer, nanotechnology and angiogenesis

Cancer is a major health problem all over the world.[1,2] According to American Cancer Society statistics, the global burden is expected to increase to 21.7 million new cancer cases by 2030.[3] According to the report published by Forbes (Pharma & Healthcare) in May 2015, the global market value for cancer has reached $100 billion and is expected to increase to $147 billion by 2018.[4] Cancer therapies involve surgery, radiation therapy, chemotherapy, immunotherapy, photodynamic therapy (PDT), cancer vaccinations, stem cell transplantations, or a combination of these. However, most of these conventional treatment strategies have several limitations and side effects.[5,6] Therefore, development of an economically convenient alternative technique for the treatment of cancers that will specifically target the tumor without harming the healthy tissue is urgently needed. In this context, nanobiotechnology can play a significant role in overcoming the limitations of conventional treatment strategies. Nanobiotechnology is the branch of nanoscience and nanotechnology that deals with the application of nanoparticles (preferably 1–100 nm) in medicine and biology. Nanotechnology has been widely used in several fields including biology and medicine due to their unique fundamental properties (small size, high surface to volume ratio and high surface energy) compared to bulk materials.[7] Recently, several investigators demonstrated nanotechnology based diagnostic (bio-imaging, diagnostics, biosensing, MRI-imaging) and therapeutic (drug delivery, antibacterial, photodynamic therapy) approaches for the treatment of several diseases (cancer, cardiovascular diseases, diabetes, Parkinson's disease, spinal cord injury, tuberculosis *etc.*).[8–13] Among several nanomaterials being used for the treatment of various diseases, this review article will focus on some special nanoparticles as anti-angiogenic (a targeted therapy that uses drugs or materials that stops angiogenesis as well as tumor growth) nanomaterials and their potential application in the treatment of cancer.

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2. Definition of angiogenesis

Angiogenesis is a complex process that helps to form new blood vessels from pre-existing vasculature.[14,15] 'Angio'

originates from the Greek word 'angeio' that means a blood vessel and 'genesis' that means production. The process of angiogenesis consists of several important steps, which include the stimulation of endothelial cells by growth factors, degradation of the extracellular matrix by proteolytic enzymes, migration and proliferation of endothelial cells, and capillary tube formation.[16,17] Naturally, a healthy body maintains the equilibrium of angiogenesis.

3. Types of vessel formation

Various types of blood vessel formation occur through several steps during angiogenesis: (i) sprouting angiogenesis, (ii) vasculogenesis and (iii) intussusception.[18–20] Sprouting angiogenesis is the most basic form of angiogenesis where new capillary vessels grow from pre-existing ones. The sprouting angiogenesis process involves several sequential steps. It is important in primary tumors as well as in metastatic tumors. During tumor angiogenesis, endothelial cells (ECs) are activated by specific angiogenic growth factors that bind to its receptors. The activated ECs release protease enzymes, which help to degrade the extracellular matrix (ECM) and the basement membrane.[18,20] This helps endothelial cells to invade into the neighboring matrix and, consequently, to proliferate and migrate through the matrix. A lumen is created by polarization of migrating cells and ultimately an immature blood vessel is generated (Fig. 1a). The stabilization of immature blood vessels requires the recruitment of mural cells and the formation of the extracellular matrix (ECM). This process of sprouting angiogenesis is tightly maintained by positive and negative regulators, the balance of which determines the level of ongoing angiogenesis.[20–22]

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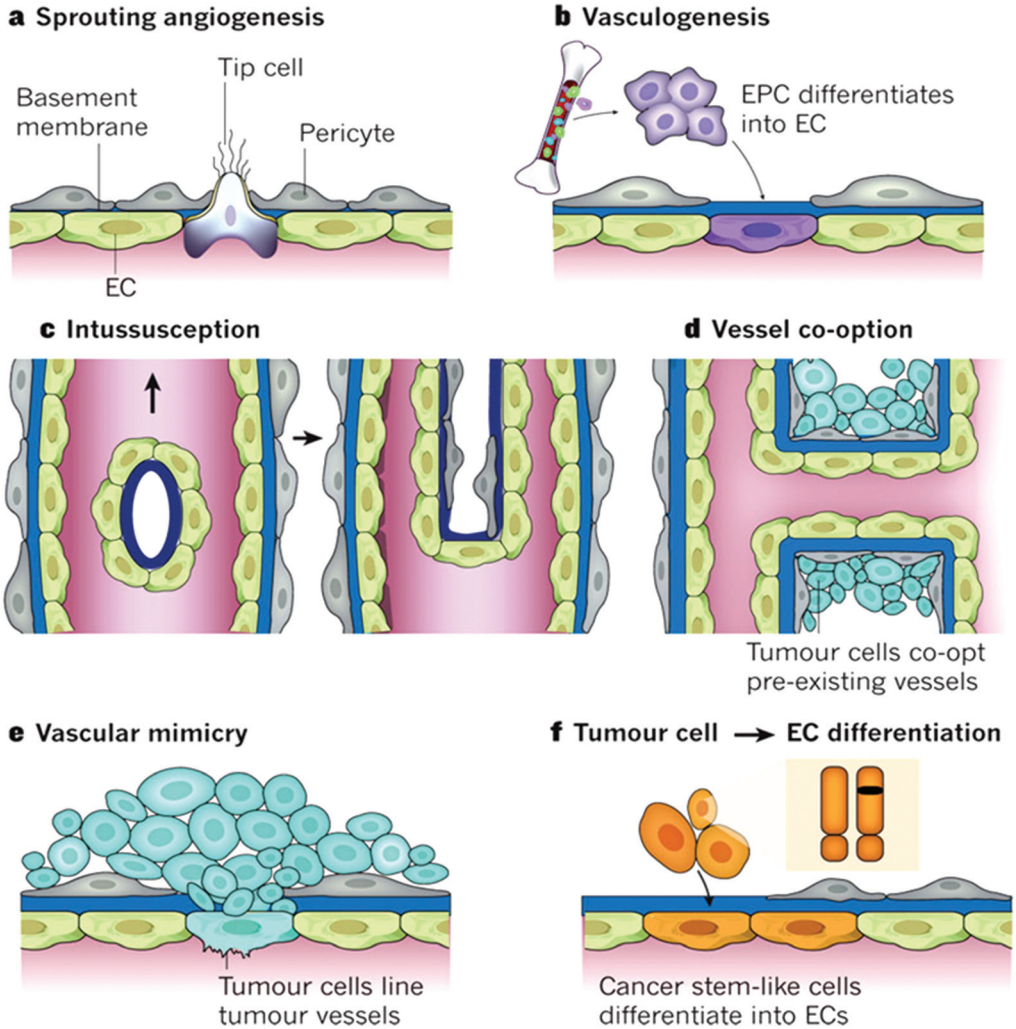

Fig. 1 Modes of vessel formation. There are several known methods of blood vessel formation in normal tissues and tumours. (a–c) Vessel formation can occur by sprouting angiogenesis (a), by the recruitment of bone-marrow-derived and/or vascular-wall-resident endothelial progenitor cells (EPCs) that differentiate into endothelial cells (ECs; b), or by a process of vessel splitting known as intussusception (c). (d–f) Tumour cells can co-opt pre-existing vessels (d), or tumour vessels can be lined by tumour cells (vascular mimicry; e) or by endothelial cells, with cytogenetic abnormalities in their chromosomes, derived from putative cancer stem cells (f). Unlike normal tissues, which use sprouting angiogenesis, vasculogenesis and intussusception (a–c), tumours can use all six modes of vessel formation (a–f). Reprinted with permission from ref. 18. Copyright (2011) Nature Publishing Group.

The vascular system initiates from two basic processes: (i) vasculogenesis and (ii) angiogenesis.[23] Vasculogenesis refers to differentiation of endothelial precursor cells (known as angioblasts) into endothelial cells (ECs) in combination with the generation of a primitive vascular network (Fig. 1b).[24,25] Initially, it was believed that vasculogenesis is restricted to early embryogenesis and does not happen in adults, whereas angiogenesis happens in both the budding embryo and postnatal life. However, a recent study says that both processes are seen during embryonic and adult growth processes.[23] The process of vasculogenesis during embryo development involves some essential steps, namely (i) formation of the angioblasts from the mesoderm, (ii) gathering of angioblasts into vascular structures, (iii) the creation of vascular lumens, and (iv) the organization of continuous vascular networks.[25,26]

Intussusception is a type of angiogenesis; it is known as splitting angiogenesis by which new blood vessels are formed from pre-existing vessels (Fig. 1c). It is a novel process of blood vessel formation and remodeling. Djonov *et al.* demonstrated that the process of intussusception plays a significant role both in early capillarization and in network restoration and development of larger vessels.[19] Furthermore, tumor vasculature tends to use another three different modes of vessel formation: (i) vessel co-option, (ii) vascular mimicry and (ii) EC differentiation from cancer stem cells.[14,15,18,27–29] It is needless to mention that different types of angiogenesis should be discussed briefly before a detailed discussion of

the application of angiogenesis/anti-angiogenesis in cancer therapy.

3.1. Significance of angiogenesis

The process of angiogenesis is very important for organ growth and repair. Angiogenesis occurs throughout life during physiological processes (embryonic development, the menstrual cycle). Disrupted balance of angiogenesis provides immune disorders, malignancy, ischaemic, chronic inflammatory disorders and infectious diseases.[15,27,30] Hence, the overall balance inside the human body is maintained by both pro- and anti-angiogenic signals.[14] Pro-angiogenic molecules are utilized for the treatment of wound healing and cardiovascular and ischemic related diseases using angiogenic therapy. On the other hand, anti-angiogenic molecules are used for the treatment of cancers and blindness. Hence, angiogenesis research including pro- and anti-angiogenic molecules will provide a new dimension for the treatment of several diseases in the near future.

4. Stimulation for angiogenesis: application in medicine

It has already been discussed that endothelial cell proliferation and migration are critical steps in the angiogenic process and several growth factors act as pro-angiogenic agents that regulate these key steps of angiogenesis.[16,17,31] Some important growth factors (VEGF: vascular endothelial growth factor, FGF: fibroblast growth factor, HGF: hepatocyte growth factor, G-CSF: granulocyte colony stimulating factor, angiopoietin (Ang1 and Ang2), insulin like growth factor, leptin, interleukin 8 (IL-8), ephrin, integrins)[32,33] and their basic angiogenic functions are tabulated in Table 1 in detail.

Many growth factors (VEGF-A or bFGF) increase blood flow in ischemic tissues *via* formation of collateral blood vessels. Therefore, therapeutic angiogenesis of these growth factors have been used for the treatment of cardiovascular and other diseases (coronary ischaemia, myocardial ischaemia, peripheral ischaemia, peripheral vascular disease, limb ischaemia, ischaemic heart disease, coronary revascularization, retinal ischemia and wound healing).[33,57] Phase-I/II clinical trials have been conducted with these growth factors or other proteins and found initially successful therapeutic results.[32,36,58] However, the angiogenic therapy for the treatment of cardiovascular related diseases using VEGF or bFGF is associated with several limitations that include thrombosis, fibrosis, non-specific angiogenesis and growth of malignant tumors. Therefore, angiogenic therapy in patients with unknown malignant tumors or diseases plays an important pathogenic role.[59] Extensive research is in progress to develop pro-angiogenic molecules as effective treatment strategies for cardiovascular and ischemic related diseases.

5. Tumor angiogenesis

In 1971, Judah Folkman and his group suggested angiogenesis dependent tumor growth and metastasis.[27,60] It is well established that a primary tumor or metastasis can grow to the size of approximately 1–2 mm^3 obtaining sufficient supply of oxygen and nutrients by diffusion. Tumor growth beyond this size needs vascularization by means of angiogenesis. Hence, the tumor switches to an angiogenic phenotype ("angiogenic

Table 1 Stimulators of angiogenesis

Angiogenic stimulator	Functions	Ref.
VEGF	Inducer of angiogenesis and lymphangiogenesis.	34 and 35
FGF	Regulates endothelial cell proliferation, migration and differentiation.	35
HGF	Stimulates cell growth. Useful for the treatment of critical limb ischemia.	36
Ang1 and Ang2	Stimulate the matured vessel formation and regulate angiogenesis.	37
PDGF	Stimulates angiogenesis and regulates cell growth and division.	38
IGF	Stimulates angiogenesis and myogenesis and induces nerve regeneration.	39
Endoglin	Stimulates endothelial cell proliferation, extracellular matrix production and TGF-β/ALK1 signal transduction.	40
Interleukin 8	Stimulates endothelial cell proliferation, survival and matrix metalloproteinases.	41
Thyroxin	Stimulates early coronary angiogenesis.	42
VE-cadherin	Stimulates endothelial junctional molecules.	43
G-CSF	Helps in endothelial cell proliferation and acts as a neuro-protective agent.	44
Integrins	Promote cell attachment and stimulate cell migration.	45
Ephrin	Helps in vascular development and angiogenic remodeling and also determines the formation of arteries or veins.	46
eNOS	Stimulates angiogenesis *via* the eNOS signaling cascade.	47
TGFbeta	Induces angiogenesis through VEGF-mediated apoptosis. Plays a dual role as a tumor suppressor in early stages and as a tumor promoter in the late stages of tumor progression.	48 and 49
YKL40	Angiogenic factor to promote tumor angiogenesis and plays a role in radioresistance and progression of glioblastoma.	50, and 51
HIF1α	Regulates tumor angiogenesis and invasion.	52
HDGF	Plays vital roles in cancer cell transformation, angiogenesis, apoptosis and metastasis.	53
Notch/DLL4	Negative regulator of tumor angiogenesis and upregulated in tumor vasculature in cancer progression.	54, and 55
Semaphorins	Anti-angiogenic agents, stimulate tumor angiogenesis.	56

switch") and attracts blood vessels from the nearby stroma. This process is controlled by a variety of pro- and anti-angiogenic factors, a requirement for further outgrowth of the tumor.[20] Up-regulation of angiogenic stimulators with the down-regulation of inhibitors can lead the angiogenesis transduction pathway that helps in the growth of the tumor.[61] Thus angiogenesis plays a crucial role in the tumor cell growth, metastasis, and survival and progression of cancer. Ischemic or hypoxic conditions can stimulate the angiogenesis process in certain cases where tissue damage occurs. It is hypothesized that blocking of angiogenesis could be an effective strategy to reduce the tumor growth.[60] However, aggressive IDH1 mutated glioblastoma can expand beyond 2 mm^3 in volume without neovessels. As a result, the blood brain barrier (BBB) is destroyed. This can lead to uncontrolled trafficking of molecules between intracerebral and extracerebral blood vessels systems.[62]

6. Anti-angiogenic therapy: mechanism and conventional treatments, and chemical inhibitors

The angiogenesis process offers the blood supply to cancer cells and contributes to tumour progression, invasion and metastasis. It is well established that a diffusible angiogenic substance (TAF: tumor angiogenesis factor) secreted by tumors stimulates endothelial cell proliferation and migration in the host capillary blood vessels.[63,64] Pre-cancerous tissue becomes cancerous after acquiring the angiogenic capacities. It is now accepted that the endogenous balance between pro-angiogenic and anti-angiogenic molecules is tipped in favor of the 'angiogenic switch'.[65] In fact, tumors secrete several pro-angiogenic growth factors. Among them VEGF is a key tumor-derived pro-angiogenic factor that is involved in multiple functions (stimulation of angiogenesis, vasculogenesis, inflammation and vascular permeability).[66] The angiogenesis research revolutionized the drug development for cancer and other biomedical applications. The discovery of anti-angiogenesis (blockage of blood supply) that results in the inhibition of cancer cell growth is useful for the treatment of cancer. For example, VEGF is highly over-expressed in a variety of tumors. The main mechanism of action of anti-angiogenic drugs involves the attachment of drugs with VEGF that keeps away the VEGF from the VEGFR, which is mainly responsible for triggering the growth of new blood vessels. Several angiogenic inhibitors have been discovered and clinically applied with success. FDA approved bevacizumab (avastin: Genentech) in the year 2004 for the treatment of advanced metastatic colorectal cancer and glioblastoma.[67] Avastin, an anti-VEGF monoclonal antibody, binds to VEGF and inhibits the activation of VEGFR by restricting its attachment with VEGFR. Again, sorafenib, sunitinib and pazopanib are FDA approved small molecule inhibitors of the VEGFR-2 tyrosine kinase that can control the growth of tumors and angiogenesis.[67–70] Other than angiogenic inhibitors, a new class of molecules have been discovered that act as a 'vascular-disrupting agent' (VDA) by generating acute vascular occlusion and disruption of blood flow in the tumor.[71] Most of the new class of molecules [ZD6126, ABT-571, tubulin-binding agents like combretastatin, MN-029, AVE8062, and the flavonoid 5,6-dimethylxanthenone-4-acetic acid (DMXAA)] are in clinical trials.[71–73] Clarke *et al.* elaborately demonstrated the various angiogenesis inhibitors (monoclonal antibody inhibitors, receptor tyrosine kinase inhibitors, inhibitors of endothelial cell proliferation, inhibitors of integrin's pro-angiogenic activity, matrix metalloproteinase inhibitors, vascular targeting inhibitors), their target and clinical status (Phase-I–Phase-III).[74] Endogenous anti-angiogenic factors (endostatin induces apoptosis in endothelial cells and inhibits their migration, and angiostatin inhibits VEGF and bFGF; interferon-alpha shows an anti-angiogenic effect by inhibiting the migration of endothelial cells) are useful for the inhibition of tumor growth. Thalidomide is an anti-angiogenic drug that inhibits angiogenesis by reducing the levels of VEGF, COX-2, bFGF and tumor necrosis factor

Table 2 Anti-angiogenic drugs for the treatment of tumor

Anti-angiogenic drugs	Mechanism of action	Cancer types	Ref.
Avastin	Anti-VEGF monoclonal antibody	Advanced metastatic colorectal cancer and glioblastoma	75
Sunitinib	Acts as multi-TKI that targets VEGFR-1–3, PDGFR	Kidney cancer and neuroendocrine tumors	76 and 77
Sorafenib	TKI which targets VEGFR-2, -3, Flt-3, PDGFR-β	Primary kidney cancer, RCC, liver cancer	77 and 78
Everolimus	Inhibitor of mammalian target of rapamycin (mTOR)	Kidney cancer and neuroendocrine tumors	79
Imatinib	(TKI) Selective inhibitor of Bcr/Abl	CML and GIST	80
Pazopanib	Acts as a multi-targeted receptor tyrosine kinase inhibitor	Kidney cancer and soft tissue sarcoma	81
Axitinib	Second generation inhibitor of VEGF-1, -2, and -3	Renal cell carcinoma	82
Denibulin (MN-029)	Vascular-disrupting agent (VDA) and reversibly inhibits microtubule assembly	Solid tumors	72
ZD6126	Vascular targeting agent and VDA	Metastatic renal cell carcinoma and metastatic colorectal cancer	73
ABT-571	VDA and acts as a antimitotic agent	Non-small cell lung cancer	71 and 83
Ombrabulin (AVE8062)	VDA	Advanced-stage soft-tissue sarcoma and head and neck squamous cell carcinoma	71 and 83

(TNF-α). The use of angiogenesis inhibitors could be useful as a new promising treatment strategy for cancer research. A list of anti-angiogenic drugs and their mechanisms of actions is provided in Table 2.

7. Limitations of conventional anti-angiogenic therapy

Conventional anti-angiogenic therapy for cancer has several limitations which can create problems for a successful therapeutic strategy. Recently, the anti-angiogenic therapy by obstructing the VEGF pathway has become the most important and widely accepted approach for the treatment of cancer. However, the clinical usage of this modality is still inadequate because of several issues such as adverse effects, toxicity, acquired drug resistance, and non-availability of valid biomarkers.[84,85] Though Bevacizumab (Avastin), the first (VEGF) targeted anti-angiogenesis drug, was clinically approved for cancer therapy, the failure of this therapy is due to the development of inherent/acquired resistance. This major challenge led to an increase of the understanding of VEGF-independent angiogenesis.[77] Scientists have found that VEGF-A may be replaced by other VEGF family gene members like VEGF-C or VEGF-D which bind to and activate the VEGFR.[86] Also, VEGF-A may be replaced by different angiogenic cascades.[86] Loges *et al.* nicely demonstrated the various mechanisms of resistance to anti-angiogenic therapy and proposed the improvement of third-generation anti-angiogenic drug candidates.[87] Malignant cells often contribute to generating resistance against the anti-angiogenic therapy through several ways. 'Some of these mechanisms are attributable to the tumor cells themselves, but malignant cells also seem to highjack their microenvironment to stimulate escape from VEGF-targeted therapy'.[87] Hence normalization of the microenvironment helps to develop third generation anti-angiogenic drugs. Tumors can switch between different modes of vascularization such as sprouting angiogenesis, vasculogenesis, and vessel cooption and vascular mimicry to ensure sufficient nutrition to acquire drug resistance.[87] It has been found that permeability of neovessels in glioblastoma (GBM) is abnormal in character compared to the normal brain blood vessels resulting in vasogenic edema. This abnormal permeability of brain blood neovessels is due to the abnormal morphology of the neovascular 'front' during hypoxic condition which helps in the secretion of small peptides and other angiogenic factors.[62,88] Besides this, malignant cells can produce multiple pro-angiogenic factors/cytokines and also can amplify the genes associated with angiogenesis during the tumor progression and metastasis[89] resulting in the acquisition of drug resistance.

Besides this, anti-angiogenic therapy itself may suggest an escape mechanism by upregulating various growth factors responsible for rescuing tumor vascularization. For example, in tumor bearing mice, VEGFR2 blockade upregulates various pro-angiogenic factors (VEGF, FGF, PIDF, angiopoietin-1 and others).[87,90] Similarly, during VEGF targeted therapy in glioblastoma (one of the most aggressive angiogenic tumors) patients, plasma levels of FGF-2, SDF-1 are found to increase upon disease progression.[91] Apart from this, several preclinical and clinical studies demonstrated the enhanced invasiveness and metastasis of glioblastoma upon blockade of VEGF using inhibitors or drugs.[92,93] Other escape theories include the overgrowth of 'hypoxic resistant' and angiogenesis independent cancer cells.[94,95] It has to be noted that the hypoxic tumor condition also helps to secrete several pro-angiogenic factors (G-CSF and SDF-1) by vessel pruning. Other than hypoxia, the release of 'cytokine storm' by healthy tissue also promotes a 'pseudo-inflamed' state that induces tumor progression, extravasations and metastatic lodging.[96,97] Again, low levels of oxygen in the hostile hypoxic tumor environment help in tumor invasiveness to a distant malignant site.[98,99] Importantly, glioblastoma (GBM) cancer stem cells (CSC), which help in the recurrence of metastasizing cancer cells, can become hypoxic tolerant and reside in hypoxic tumor regions, which can sustain self-renewal and maintain the bulk tumor.[100] Additionally, hypoxic conditions help in the recruitment of endothelial progenitor cells (EPCs) and recruited bone marrow-derived circulating cells (RBCCs) towards the tumor vasculature, which can indirectly promote tumor angiogenesis by the secretion of several angiogenic cytokines.[87,101]

The other important mechanism for resistance and escape is the activation of the HGF/MET pathway. HGF are mainly produced by bone marrow-derived cells (BMDCs). Expression of the HGF receptor c-Met in a murine xenograft tumor model was primarily found in vascular endothelial cells and not in tumor cells. This suggests a role for HGF-stimulated c-Met signaling in endothelial cells to bypass anti-VEGF therapy.[100] Eckerich *et al.* demonstrated that hypoxia can prompt c-Met expression in glioma cells and improve SF/HGF-induced cell migration.[102] Blockade of angiogenesis helps in the increase of c-Met transcription and subsequent invasion of glioblastoma tumor cell lines. Upon hypoxic exposure, induction of c-Met expression augments HIF-1α levels, which can cause tumor cell migration *via* HGF stimulation. VEGF can also directly and negatively modulate the activity of c-Met through the interaction of the c-Met–VEGFR2 complex on tumor cells *via* a hypoxia independent mechanism. Thus, VEGF blockade can enhance c-Met phosphorylation and subsequent migration and invasion in murine and human GBM.[100,102]

Another key limitation of anti-angiogenic therapy combined with radiation is the decrease in the response to radiotherapy upon reduced oxygen content or under tumor hypoxic conditions. Fenton *et al.* demonstrated that tumor hypoxia acquires at a distant location from the perfused blood vessels after 24 h of post irradiation, suggesting a decrease in oxygen consumption at 24 h. This can indirectly promote tumor resistance and tumor cell invasion.[103] Thus tumor hypoxia can limit the actions of radiotherapy, which may be overcome by the delivery of oxygen to the hypoxic area.[104] However, the underlying physiological mechanisms behind the limitation of radiotherapy under hypoxic conditions remain somewhat

unclear and need thorough mechanistic studies. Other limitations include side effects of anti-angiogenic chemotherapeutic drugs to other organs and tissues, high blood pressure, risk of pregnancy and surgery, bleeding or damage in the digestive tract and high cost.

8. Alternative anti-angiogenic therapy (nanomedicine approach)

The angiogenesis research revolutionized the development of anti-angiogenic drugs that inhibit cancer cell growth, useful for the treatment of cancer. The anti-angiogenic approach inhibits either the binding process between pro-angiogenic growth factors and their corresponding receptors or the activity of proteolytic enzymes of the extracellular matrix. The toxicity of most of the anti-angiogenic drug molecules (small molecules) or instability of anti-angiogenic proteins requires developing a new formulation in an appropriate delivery system.[63]

In this context, nanomedicine plays a pivotal role. The unique properties of nanomaterials (small size, high surface to volume ratio and high surface energy compared to bulk materials) can be used to more effectively deliver the active components to target sites.[105] Nanotechnology is a platform where researchers from different backgrounds of chemists, physicists, biologists, pharmacologists, materials science scientists, medical doctors, and engineers can work together for interdisciplinary and integrative research. The successful translation of nanotechnology based therapeutic and diagnostic tools from the laboratory to the clinic and *vice versa* needs extensive multidisciplinary research.[106,107] Recently, several investigators including our group demonstrated the nanotechnology based diagnostic (bio-imaging, diagnostics, biosensing, MRI-imaging) and therapeutic (drug delivery, antibacterial, photodynamic therapy) approaches for the treatment of several diseases including cancer, cardiovascular diseases, diabetes, Parkinson's disease, spinal cord injury, tuberculosis *etc*.[8–13] In general, engineered, modified and fabricated nanomaterials can overcome the limitations that are found in conventional treatment strategies. Sometimes, active anti-angiogenic drug molecules conjugated with nanoparticles containing targeting ligand can improve the therapeutic efficacy of anti-angiogenic molecules in a tumor microenvironment. Since conventional anti-angiogenic therapy has several limitations,[85] we believe that the nanomedicine approach could be used as an alternative treatment strategy for cancers using the anti-angiogenic properties of nanomaterial or nanoparticle based drug delivery systems. Recent reports demonstrate both pro- and anti-angiogenic properties of nanomaterials. Several groups including ours have demonstrated the pro-angiogenic properties of different nanomaterials including europium hydroxide nanorods (EHNs), zinc oxide nanoflowers, copper nanoparticles (CuNPs), carbon nanotubes (CNT), graphene oxides (GO), and cerium oxide nanoparticles (NCe).[108–115] On the other hand, nanoparticle based anti-angiogenic drug delivery systems were developed to suppress tumor angiogenesis.[116–118] Furthermore, the unique properties of nanoparticulate systems (nanoparticles, liposomes, polymeric micelles, *etc*.) can be used to target endothelial cells and the efficient delivery of anti-angiogenic agents.[63] Various nanoparticles show the targeting ability. Nanoparticles conjugated with the corresponding targeting agent can target VEGF and its receptors, fibroblast growth factor and its receptors, EGFRs, MMPs, tubulin function and so on. These targeted drug

Table 3 Anti-angiogenic nanomaterials and their therapeutic applications

Serial no.	Nature of nanoparticles	Anti-angiogenic activity	Ref.
1	Cerium oxide	Ovarian tumor model	119
2	Fullerenol (F) and its conjugates	Zebrafish and murine tumor angiogenesis models	113
3	Chitosan	Inhibition of hepatocellular carcinoma xenografts	120
4	Fullerenic	Inhibition of MCF-7 breast tumor model	121
5	Tetrac	Inhibition of human renal cell carcinoma xenografts	122
6	Biosynthesized AgNPs	Anti-angiogenic activity	123
7	Carbon	Inhibition of glioblastoma multiforme	124
8	Gold	Anti-angiogenic activity in HUVEC	125
9	Gold	Anti-angiogenic activity in a CAM model	126
10	Functional peptide with AuNPs	Inhibition of *in vitro* angiogenesis	127
11	GO & rGO	Switchable angiogenic and anti-angiogenic activities	114
12	Gold	Ovarian cancer in a mouse model	128
13	Biogenic AgNPs	Anti-angiogenesis effect on CAM	129
14	Cuprous oxide	Inhibition of angiogenesis *via* down-regulation of VEGFR2 expression	130
15	Carbon nanomaterials and their derivatives	Anti-angiogenic activity through the down-regulation of KDR	131
16	Silicate	Anti-angiogenic effect on retinal neovascularization	132
17	NAMI-A-loaded mesoporous silica	Inhibition of angiogenesis by the production of ROS	133
18	Perfluorocarbon	Diagnosis and treatment of atherosclerosis	134
19	Magnetic mesoporous silica-based siRNA	Orthotropic ovarian cancer therapy	135
20	Peptide	Anti-angiogenic therapy in a glioma model	118
21	AuNPs & AgNPs with heparin	Inhibition of FGF2-induced angiogenesis	136
22	Gold	Anti-angiogenic activity through heparin-binding glycoproteins	137
23	Biosynthesized AgNPs	Inhibition of VEGF- and IL-1-induced vascular permeability in PRECs	138
24	Perfluorocarbon	Anti-neovascular efficacy in the rabbit Vx2 cancer model	139

delivery systems allow them to deliver both anti-angiogenic molecules and anti-cancer drugs, facilitate drug penetration into extravascular tumor tissue and consequently increase the therapeutic efficacy and reduce the systemic toxicity. There are limited reports of therapeutic anti-angiogenic nanoparticles (tabulated in Table 3) that could be useful as effective treatment strategies for cancers.

9. Anti-angiogenic nanomaterials

Recently researchers developed and designed several nanoparticles showing anti-angiogenic properties. Nanotechnology based anti-angiogenic therapy is becoming a promising approach for cancer treatment. These anti-angiogenic nanoparticles could be useful as an alternative treatment strategy for the treatment of various cancers using anti-angiogenic therapy. Examples of a few anti-angiogenic nanomaterials and their therapeutic applications are described below.

9.1. Cerium oxide nanoparticles

Cerium oxide nanoparticles have vast applications in various biomedical applications for the treatment of several diseases including cancer, diabetes, macular degeneration, Alzheimer's disease, atherosclerosis, stroke *etc*.[140,141] Giri *et al.* developed and engineered nanoceria (NCe), nanoparticles of cerium oxide that show anti-oxidant properties as well as anti-angiogenic activity, based on several *in vitro* and *in vivo* assays.[119] The authors discussed that treatment of HUVEC with nanoceria showed inhibition of VEGF165-induced HUVEC proliferation (analyzed by quantification of DNA synthesis using [3H]-thymidine incorporation assay), capillary tube formation, phosphorylation of VEGFR2 (Y1175 and Y951) observed by immunoblot analysis, and activation of MMP2. Investigators showed that nanoceria possessing anti-angiogenic properties regulates ovarian tumor growth in a preclinical mouse model (nude mice) of ovarian cancer. The tumors were developed in nude mice by injecting A2780 ovarian cancer cells intra-peritoneally. Inhibition of tumor growth was accompanied by a reduction of angiogenesis, observed by reduced tumor weight, histopathological analysis (H&E staining), reduced Ki-67 and CD31 staining and specific apoptosis of vascular endothelial cells (Fig. 2). Authors demonstrated that nanoceria reduce the growth factor mediated migration and invasion of SKOV-3 cells, VEGF165 induced proliferation, capillary tube formation and stimulation of VEGFR2 and MMP2 in HUVEC cells. NCe inhibited tumor growth in a mouse model due to anti-angiogenic activity, based on the reduction of CD31 staining and specific apoptosis of vascular endothelial cells. Inductively coupled plasma mass spectroscopy (ICP-MS) and transmission electron microscopy (TEM) were utilized to find out the accumulation of NCe in tumors isolated from the NCe treated group. Based on the collective results, the research group demonstrated that nanoceria could be used as an effective anti-angiogenic therapeutic agent for the treatment of ovarian cancer. Another research group designed nanoceria functionalized with heparin and demonstrated that heparin-nanoceria reduce endothelial cell proliferation indicating the anti-angiogenic properties that could be useful for the treatment of cancer.[142] They showed that NCs were localised mostly in the cytoplasm, while heparin-nanoceria were localized in both the cytoplasm and lysosomes. In another study, Alpaslan *et al.* functionalized the nanoceria with dextran and they found that dextran coated NCs significantly inhibit the proliferation of bone cancer cells (osteosarcoma cells) under slightly acidic conditions (pH 6) compared to physiological and basic pH values (pH 7 and pH 9). However, under slightly acidic conditions, toxicity was not observed while non-cancerous cells were incubated with these nanomaterials. Taken together, the nanoparticles could be useful for the treatment of bone cancer.[143] Other reports suggest that nanoceria or nanoceria conjugated with chemotherapeutic drugs show anti-angiogenic properties that could be used for the treatment of cancers.[144,145]

9.2. Gold nanoparticles (AuNPs)

Gold nanoparticles and their conjugates were extensively used in various biological and medicinal applications (targeted drug/gene/antigen/siRNA/shRNA delivery, immunoassays, clinical chemistry genomics, biosensorics, photothermolysis of cancer cells and tumors, optical bioimaging *etc*.) due to their unusual physico-chemical properties, tunable size, small dimensions, low toxicity, long history of use in medicine, and biocompatibility.[146–148] Mukherjee *et al.* for the first time demonstrated that 5 nm of spherical bare gold nanoparticles (AuNPs) shows anti-angiogenic properties.[137] Initially, authors reported the synthesis and characterization of sodium borohydride reduced AuNPs and demonstrated the inhibition of VEGF165-induced proliferation of HUVEC cells.[125] As we know, vascular permeability factor/vascular endothelial growth factor 165 (VPF/VEGF-165), a 45 kDa heparin-binding endothelial cell (EC) specific mitogen, promotes angiogenesis that plays a significant role in pathological neovascularization (rheumatoid arthritis, neoplastic disorder, chronic inflammation). It is well established that the heparin binding growth factor VEGF165 and a non-heparin binding growth factor, VEGF121, activate the cell surface kinase receptor (KDR), and bind to vascular endothelial growth factor receptor 2 (VEGFR-2), and thus induce proliferation of endothelial cells (HUVEC). The authors showed that the AuNPs inhibit the activity of VEGF165 but do not interfere in the activity of VEGF121.[125] The authors investigated whether the interaction between AuNPs and VEGF165 primarily happens through the heparin binding domain of the protein. Since VEGF121 does not have a heparin binding domain, its activity is not reduced by AuNPs.[137] Gold nanoparticles bound to VEGF165 through the heparin binding domain inhibited the activity of KDR and prompted the anti-angiogenesis cascade. The research group also showed that bare AuNPs bound to basic fibroblast growth factor (bFGF), another crucial cell mitogen and mediator of angiogenesis, and inhibits its activity. The AuNPs inhibit the fibroblast cell proliferation along with VEGF-induced permeability and angiogenesis towards *in vivo*.[137] They have investigated the

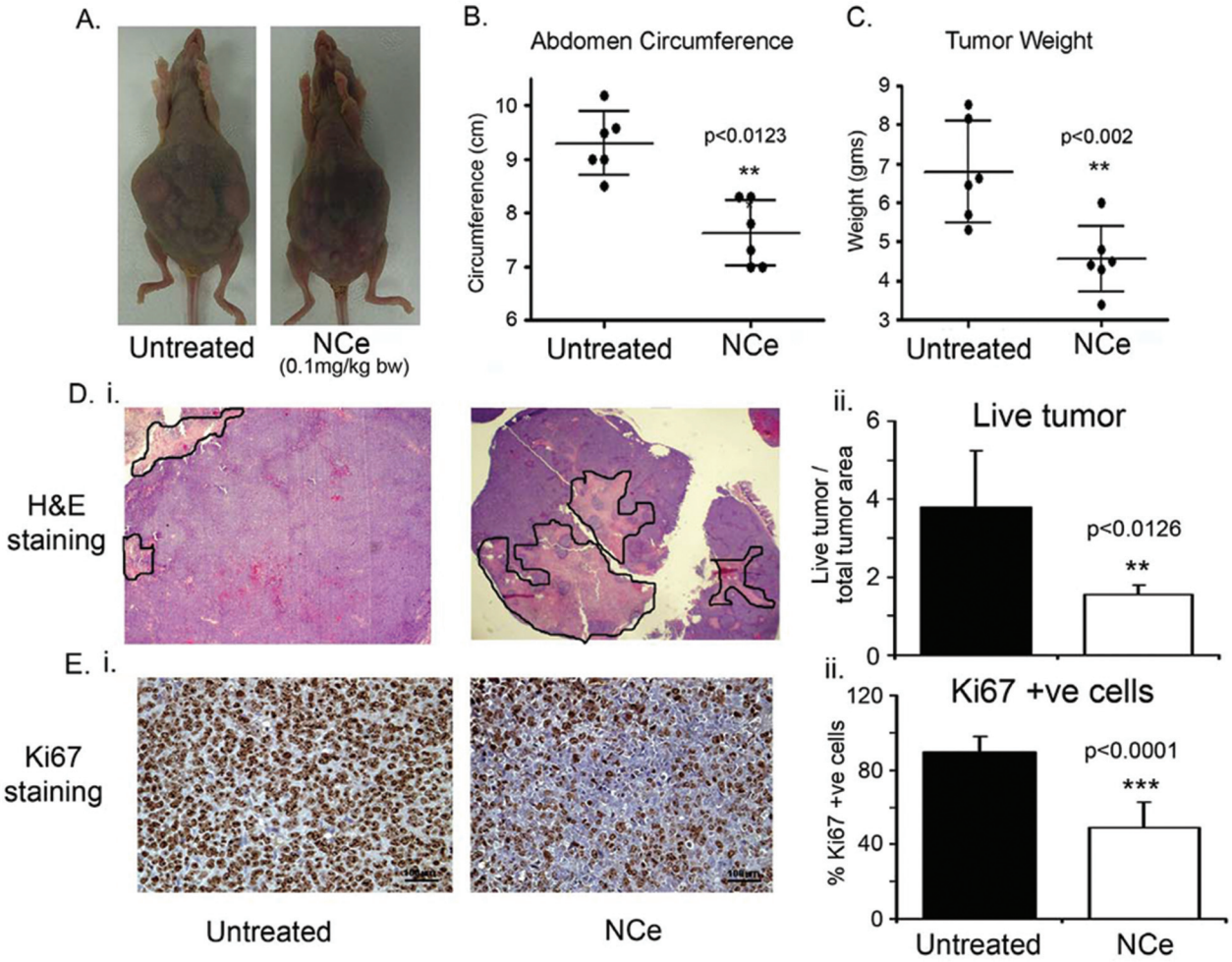

Fig. 2 NCe treatment inhibited ovarian tumor growth *in vivo*. (A) Gross morphology of a representative mouse with tumors at day 30 (n = 6). (B) Cumulative abdominal circumference at the end of the study. (C) Excised tumor weight from vehicle (PBS) treated and NCe groups (0.1 mg per kg bd wt; every third day). Results are shown as the mean 6 S.D. of six individual animals. **p, 0.01 NCe treated group compared to untreated group using a two-tailed Student's t-test (Prism). (D) (i) Representative H&E (×20) photomicrographs exhibiting live (purple) and necrotic (pink, encircled) areas in untreated and treated xenografts. (ii) Graphical representation of viable tumor size measured as described in the Material and methods section. (E) (i) Representative Ki-67 staining (×200) of excised A2780 xenografts at day 30. (ii) Count of positive Ki-67 cells from 5 high powered fields (×400) in 3 different xenografts from each group. Counts are expressed as percentage of the control. ***p, 0.001 and **p, 0.01 NCe treated group compared to untreated group using a two-tailed Student's t-test (Prism). DOI: 10.1371/journal.pone.0054578.g004. Reprinted with permission from ref. 119. Copyright (2013) Plos.org.

efficacy of nanogold to inhibit VEGF165-induced permeability and angiogenesis in a mouse ear model. AuNP treated mice showed less edema and reduction of angiogenesis compared to mice treated with Ad-VEGF adrenoviral vector of VEGF injected mice (positive control). They efficiently correlate to their *in vivo* data (mouse ear and mouse ovarian tumor models) with *in vitro* results. Serum clinical chemistry of mice treated with AuNPs did not show any toxicity in serum levels of creatinine, blood urea nitrogen, bilirubin alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase compared to the untreated mice. Since the growth factor mediated proliferation and angiogenesis have a significant role in various pathological conditions (neoplasia, rheumatoid arthritis, chronic inflammation, and wound healing), this study will be very important for these diseases. Moreover, easy synthesis, surface modification and low production cost of nanogold would make them feasible for various biomedical applications including promising anti-angiogenic therapy for the treatment of cancers.

The same group later investigated the mechanism of anti-angiogenic properties of AuNPs.[149] They demonstrated that the size and surface charge of gold nanoparticles play an important role in pro-angiogenic heparin-binding growth factors (HB-GFs), including VEGF165 and bFGF. In order to prove that VEGF165 and bFGF were pre-incubated overnight with AuNPs of different sizes (5, 10, and 20 nm) at 4 °C and later, these were added to serum-starved HUVECs and NIH-3T3 cells. Based on the [3H]-thymidine incorporation assay,

VEGF165-induced proliferation of HUVECs was significantly inhibited by all sizes of AuNPs in a dose dependent manner (Fig. 3). The larger nanoparticles (20 nm) showed the maximum inhibition effect whereas the smallest nanoparticles (5 nm) showed the lowest effect. Dose dependent inhibition of cell proliferation in HUVEC is presented in Fig. 3(B–D). Complete inhibition of VEGF165-induced proliferation was accomplished with 1 nmol L^{-1} of 20 nm AuNPs (Fig. 3D). Similar results were seen with bFGF-induced proliferation of NIH-3T3 fibroblasts. Gold nanoparticles reduce the VEGF165 induced KDR-phosphorylation in a size dependent manner, supported by western blot analysis. The authors hypothesized that the inhibitory effect of AuNPs was due to the change in HB-GF conformation/configuration (denaturation) by nanoparticles. However, AuNPs do not change the conformations of non-HB-GFs. They demonstrated that 20 nm of negatively charged (−40 mV) AuNPs inhibited angiogenesis by electrostatic binding with positively charged heparin binding domains, which is superior to the smaller sized (5 nm and 10 nm) AuNPs, positively charged or surface modified counterparts.[149] Higher size AuNPs (20 nm) showed more binding with VEGF165 than smaller sized AuNPs, probably leading to anti-angiogenic properties. The same group also demonstrated AuNPs as potential anti-tumor and anti-metastatic agents for the treatment of ovarian cancer using two separate orthotropic models of ovarian cancer.[128]

It is well established that the inhibition of the binding interaction between growth factors (*e.g.* VEGF) and their receptors (*e.g.* VEGF-R2) reduces angiogenesis and delays tumor growth. Pan *et al.* investigated the effect of AuNPs on the interaction of VEGF with its receptor, VEGFR2, using near-field scanning optical microscope and quantum dot (NSOM/QD) imaging.[150] They observed that AuNPs inhibit the VEGF165-induced VEGFR2 and AKT phosphorylation.

Authors also demonstrated the anti-tumor activity of AuNPs in xenograft and ascites models. Furthermore, authors demonstrated the inhibition of angiogenesis in a liver tumor nude mice model, determined by CD34 immunohistochemistry that shows the reduction of microvascular density. Recently, the same group reported the inhibition of VEGF165-induced migration and tube formation of endothelial cells *via* the Akt pathway in the presence of gold nanoparticles. The results were supported by several *in vitro* assays (cell migration assay, tube formation assay, western blot analysis, CAM assay: chick chorioallantoic membrane assay *etc*.).[126] Based on the results, the anti-angiogenic properties of gold nanoparticles could be effectively utilized for the treatment of cancer.

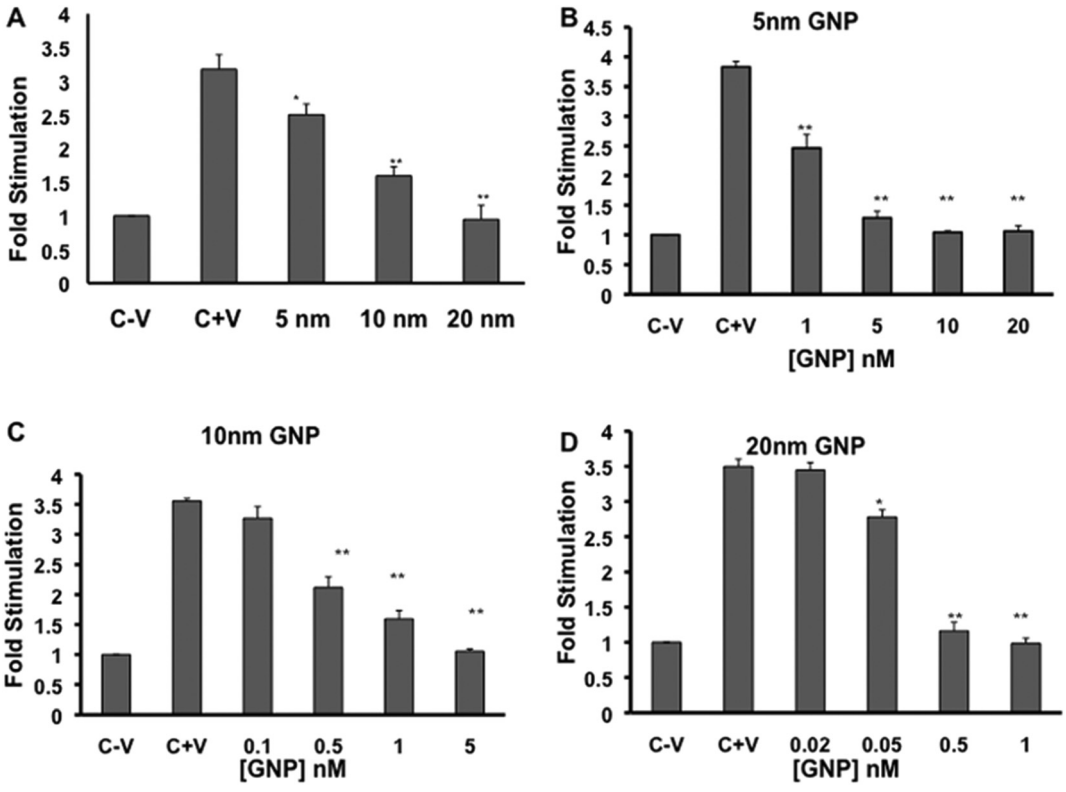

Fig. 3 Effect of gold nanoparticle core size on cell proliferation in HUVECs. [3H] thymidine incorporation is represented as fold stimulation. (A) Serum starved HUVECs were stimulated with 10 ng ml^{-1} VEGF165 that was preincubated with and without gold nanoparticles (conc = 1 nmol L^{-1}). (B–D) The effect of dose on HUVEC proliferation with 5 nm (B), 10 nm (C), and 20 nm GNPs (D). The analyses for each nanoparticle were done in triplicate and each C + V = cells stimulated with VEGF165 alone. *$p < 0.01$, **$p < 0.005$ as determined by a two-tailed Student's *t*-test. Error bars, mean ± SD. Reprinted with permission from ref. 149. Copyright (2009) Elsevier.

9.3. Silver nanoparticles (AgNPs)

Another example of important noble metal nanoparticles is silver nanoparticles (AgNPs) that show immense potential therapeutic application in biomedical applications (anti-bacterial activity, anti-cancer activity, imaging, anti-fungal, drug delivery, bio-sensing *etc*.).[151–153] Recently, several groups demonstrated the anti-angiogenic therapeutic efficacy of chemically and biologically synthesized AgNPs.[123,129,138,154] Sheikpranbabu *et al.* synthesized AgNPs using *Bacillus licheniformis* biomass as a reducing agent. The researchers investigated the inhibition of VEGF- and interleukin-1 beta (IL-1β) induced vascular permeability *via* a Src dependent pathway in porcine retinal endothelial cells (PRECs). AgNPs inhibit the VEGF- and IL-1β-induced Src phosphorylation at Y419.[138] The authors also observed the internalization of AgNPs into PREC cells using TEM. The results altogether demonstrate that bio-synthesized AgNPs could represent a potential therapeutic target to inhibit the ocular diseases including diabetic retinopathy.

Gurunathan and co-workers demonstrated the anti-angiogenic properties of biogenic silver nanoparticles, synthesized using *Bacillus licheniformis.*[123] According to their hypothesis, AgNPs show anti-angiogenic properties by inhibiting VEGF induced cell proliferation, migration, and capillary-like tube formation of bovine retinal endothelial cells (PEDF), a potent anti-angiogenic agent. Additionally, AgNPs successfully inhibit the development of new blood microvessels induced by VEGF in the mouse Matrigel plug assay through the inhibition of the activation of PI3K/Akt pathways. Fig. 4 shows the anti-angiogenic activity of AgNPs in an *in vivo* rat model. *In vivo* Matrigel plug assay in C57/BL6 mice demonstrated that AgNPs inhibit the formation of blood vessels and micro-vessels due to the

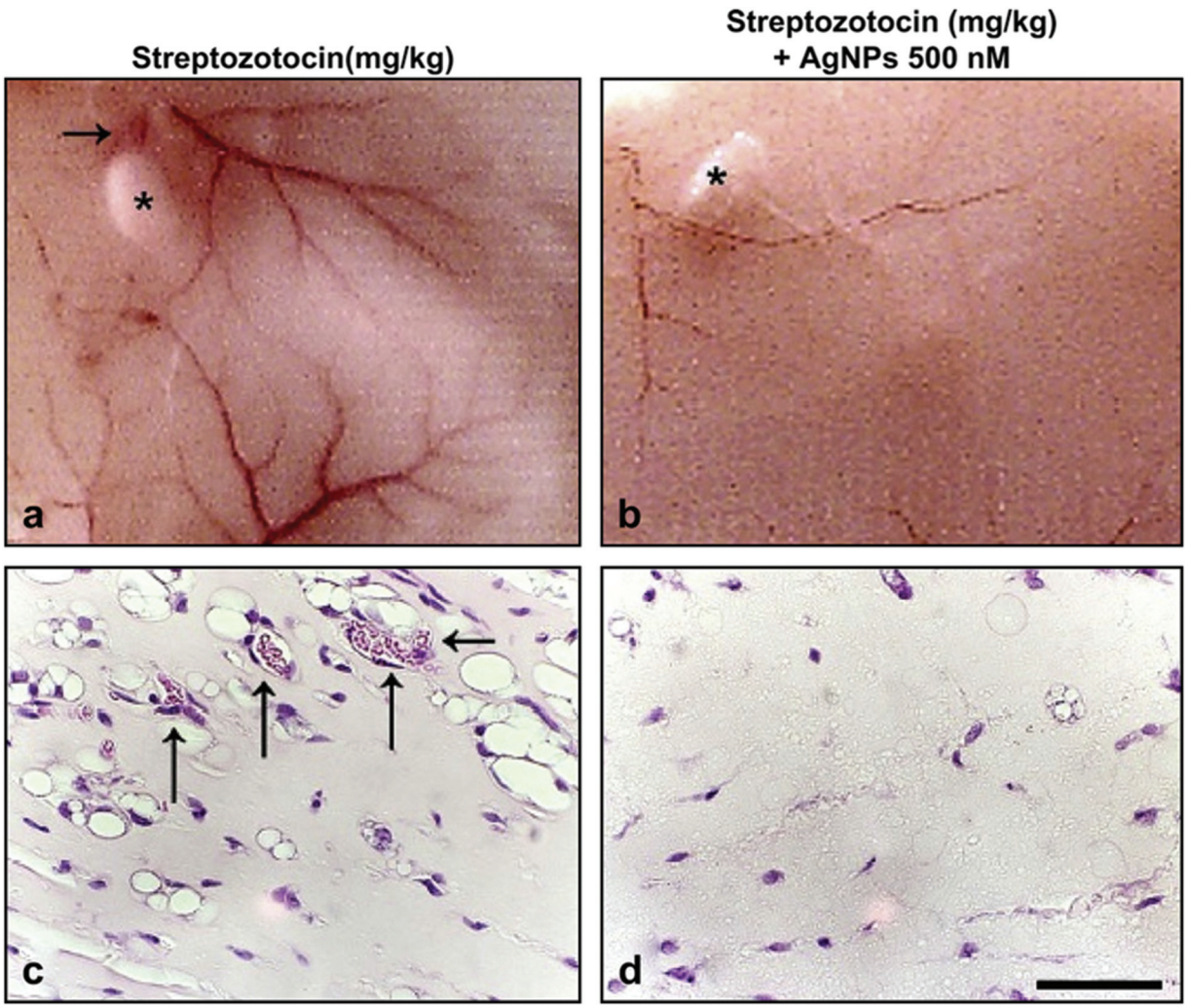

Fig. 4 Anti-angiogenic activity of AgNPs in an *in vivo* rat model. After the rats were sacrificed, tissues were photographed. Top panel: gross photographs of day 7 Matrigel implants with a skin vessel background. Representative figures show (a) streptozotocin without Ag-NPs, (b) streptozotocin plus Ag-NPs. Bottom panel: histologic sections and hematoxylin and eosin stained cross-sections showing representative photographs obtained from the sections of retina stained by hematoxylin and eosin in rats (c, d). Significant differences from the control group were observed ($p < 0.05$). Reprinted with permission from ref. 123. Copyright (2009) Elsevier.

anti-angiogenic effect of AgNPs.[123] On the other hand, administration of exogenous streptozotocin itself shows more blood vessels (angiogenic) (Fig. 4a and b). Additionally, H&E staining supports the above results (Fig. 4c and d). Their results demonstrate that anti-angiogenic properties of AgNPs can be effectively used for the treatment of cancer and other diseases. In another report, Kalishwaralal *et al.* investigated the anti-angiogenic properties of silver nanoparticles, synthesized using *Bacillus licheniformis*.[154] Authors reported that silver nanoparticles inhibit VEGF induced cell proliferation, migration, and cell survival in bovine retinal endothelial cells (BRECs) *via* the PI3K/Akt dependent pathway.[154] The inhibitory effect of AgNPs was demonstrated by induction of apoptosis, which was supported by enhancement in caspase-3 activity and formation of DNA ladders. Very recently, Baharara *et al.* synthesized AgNPs using *Salvia officinalis* plant extract through a cost effective and eco-friendly green chemistry approach. Authors investigated the anti-angiogenic properties of these nanoparticles.[129] All experiments (measurement of hemoglobin, CAM assay *etc.*) together support the anti-angiogenic properties of AgNPs. Sriram *et al.* demonstrated the antitumor activity of biologically synthesized AgNPs in Dalton's lymphoma ascites tumor model.[155] The AgNPs revealed dose dependent cytotoxicity against DLA cells that leads to induction of apoptosis through activation of the caspase-3 enzyme. Anti-angiogenesis properties of gold and silver nanoparticles conjugated with heparin derivatives were reported by Kemp *et al.*[136] Recently, our group demonstrated the multifunctional biological activities of bio-synthesized AgNPs (4-in-1 system) that could be useful as (i) an anti-cancer, (ii) an anti-bacterial, (iii) a drug delivery vehicle, and (iv) an imaging facilitator.[153]

9.4. Copper nanoparticles

Nanoparticles of copper element (cuprous oxide, copper sulphide, cupric oxides) were extensively used for various biomedical applications including cancer therapy, anti-bacterial effect, photothermal effect, and drug delivery.[130,156,157] Song *et al.* demonstrated the anti-angiogenic properties of cuprous oxide and copper nanoparticles (CuNPs), based on various *in vitro* assays in HUVEC and *in vivo* studies.[130] The CuNPs inhibit HUVEC cell proliferation, migration, tube formation, and cell cycle (arrest in S-phase) in a dose dependent manner based on the *in vitro* assays and *in vivo* angiogenesis assay. In the *in vivo* Matrigel plug assay, inhibition of *in vivo* blood vessel formation observed by CD31 staining further supports the anti-angiogenic properties of CuNPs. Furthermore, the anti-angiogenesis activity of CuNPs was accompanied by the inhibition of VEGFR2 expression at both the protein and mRNA levels in a dose and time dependent manner. In a recent study, Zhang *et al.* demonstrated that $\alpha\nu\beta3$ conjugated soft copper oleate nanoparticles ($\alpha\nu\beta3$-CuNPs) were efficiently delivered as a potent anti-angiogenic pro-drug, fumagillin.[158] This is an example of a systemically targeted drug delivery therapy using a photoacoustic contrast agent. Other than anti-angiogenic properties, some reports support the angiogenic properties of CuNPs.[112,159] For the development of blood vessel growth and muscle development, copper is a key element. However, the release of copper ions from Cu salts is toxic. The authors demonstrated that 50 ppm of CuNPs shows generation of new blood vessels, observed by CAM assay, an *in vivo* angiogenesis assay. They also investigated different pro-angiogenic mRNA gene expressions including VEGF-A, FGF-2, Myo D1, COX, PCNA *etc*. Mainly VEGF-A and FGF-2 gene expressions are elevated in the CuNPs treated pectoral muscles of embryos compared to the control group after 20 days. Thus, copper in the elemental nano form shows deviation from the angiogenic behavior in the oxide form. The same group of researchers demonstrated the positive influence of CuNPs and $CuSO_4$ on broiler chicken's performance.[159] According to researchers for postnatal growth, the *in ovo* administration of Cu colloids may ensure an efficient penetration of Cu into the embryonic tissue with long-lasting effects.

9.5. Silicate and silica based nanoparticles

Like other nanomaterials, silica and silicate based nanoparticles were used for various biomedical applications including biosensors, enzyme supporters, controlled drug release and delivery, cellular uptake *etc*.[160] These nanoparticles were also used as anti-angiogenic agents or as drug or siRNA delivery vehicles for cancer therapy, in addition to retinal neovascularization *etc*. Chen and his colleagues designed and developed the magnetic mesoporous silica-based nanoparticle (M-MSN) based siRNA (VEGF-small interfering RNA) delivery systems (M-MSN-VEGF siRNA@PEI-PEG-KALA) that were obtained after capping with polyethylenimine (PEI), grafting with polyethylene glycol (PEG) and functionalization with fusogenic peptide (KALA).[135] This delivery system showed substantial efficiency in the delivery of VEGF-small interfering RNA (siRNA) towards *in vitro* (SKOV-3 cells) and *in vivo* systems (orthotropic ovarian tumor-bearing nude female BALB/c mice). Furthermore, they showed that the magnetic core can be successfully utilized as a probe or a magnetic-imaging agent for cancer diagnostics. According to authors, significant inhibition of angiogenesis was observed by systemic administration of this nanocarrier (100 mg kg^{-1} of delivery system containing 3.5 nmol siRNA). Also no significant toxic drug responses were noticed in major organs. Immunohistological and immunoadsorbent analyses showed a decrease in VEGF expression, indicating the inhibition of angiogenesis by these nanomaterials. Overall, the authors claimed that their M-MSN-based delivery system could be useful as a potential carrier of siRNA therapeutics in ovarian cancer.

Hu *et al.* developed NAMI-A (imidazolium *trans*-imidazole dimethyl sulfoxide tetra chlororutheate) loaded and RGDK peptide modified silica nanoparticles (NAMI-A@MSN-RGD) that can be utilized for enhanced anti-angiogenic therapy in *in vitro* (HUVEC) and *in vivo* CAM models.[133] NAMI-A is well established anti-tumor and anti-angiogenic drug currently undergoing clinical investigations.[161] Fig. 5 shows the enhanced anti-angiogenic efficacy of NAMI-A@MSN-RGD, observed by wound healing or scratch assay, invasion assay and tube formation assay and compared the results with

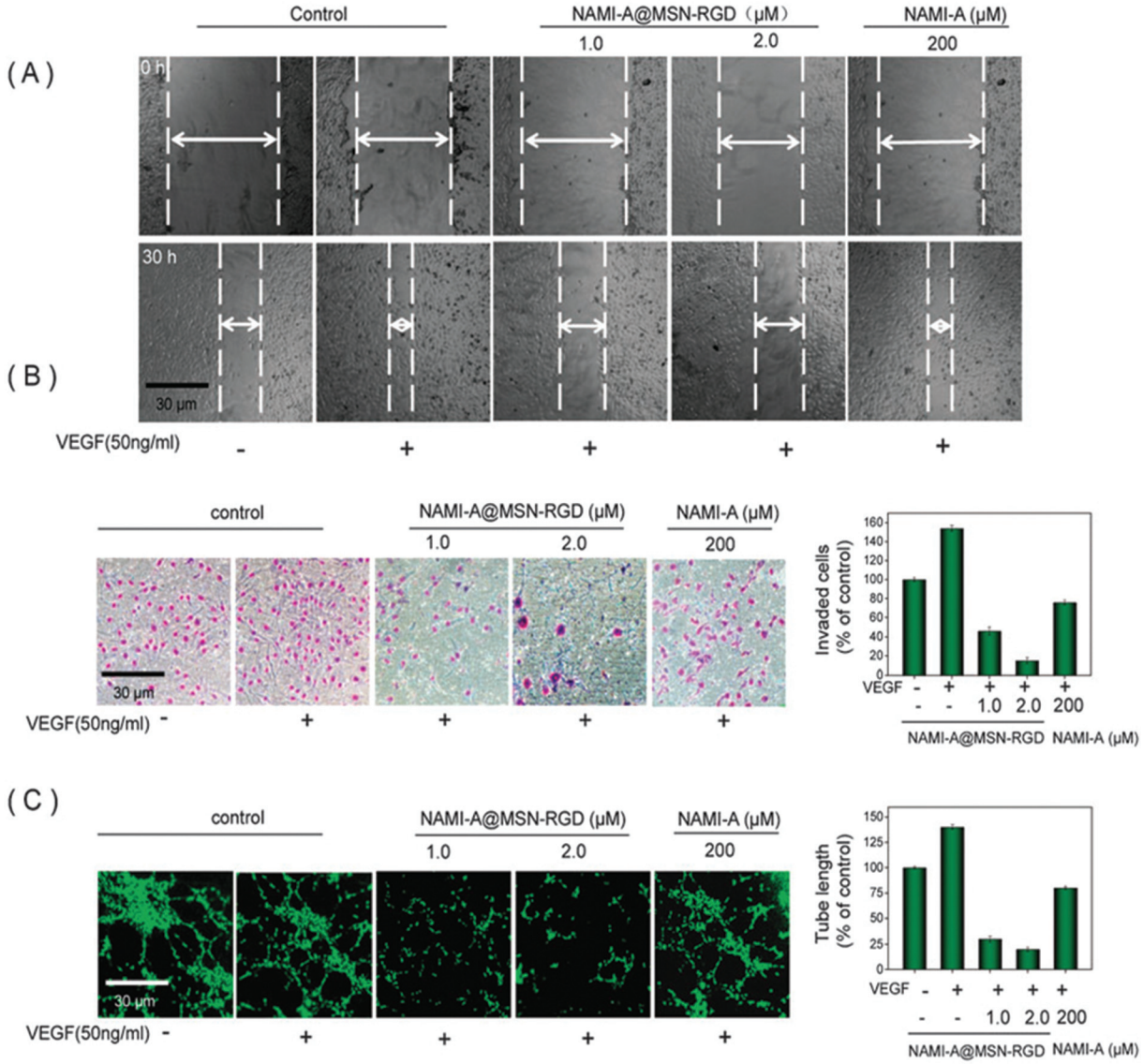

Fig. 5 (A) Anti-wounding healing assay of NAMI-A@MSN-RGD and NAMI-A on HUVEC (2×10^5 cells per ml). (B) Anti-invasion assay of NAMI-A@MSN-RGD and NAMI-A on HUVECs (5×10^4 cells per ml). (C) Anti-angiogenesis assay of NAMII-A@MSN-RGD and NAMI-A on HUVECs (5×10^4 cells per ml). The relative reduction of the width of cell healing, invaded cell numbers, and capillary tube length suggested remarkable anti-metastasis effects of NAMI-A@MSN-RGD and NAMI-A. The quantitative data were analyzed by manual counting (% of control). Reprinted with permission from ref. 133. Copyright (2015) The Royal Society of Chemistry.

pristine NAMI-A. Anti-angiogenesis of NAMI-A@MSN-RGD involves ROS mediated apoptosis that is associated with 'Sub-G1'-phase arrest in HUVEC. In another study, the anti-angiogenic effect of silicate nanoparticles (SiNPs) on the retinal neovascularization was demonstrated by Jo *et al.*[132] Anti-angiogenic effects of SiNPs were investigated by several *in vitro* assays including cell proliferation, wound migration, tube formation and *in vivo* assay such as an oxygen-induced retinopathy (OIR) model (5–10 μg mL^{-1}). Mechanistic studies revealed that the anti-angiogenic effects of SiNPs were associated with the inhibition of VEGFR-2 phosphorylation by the blocking of ERK 1/2 activation. In another study, Duan *et al.* demonstrated the induction of autophagy using SiNPs in endothelial cells and pericytes. This results consequently disrupt the endothelial cell homeostasis and impair angiogenesis.[162] FITC loaded (FITC-Si) and suramin loaded (Sur-Si) silica nanoparticles demonstrated the anti-angiogenic theranostic prospects.[163] The nanoformulation shows potential application in future anti-angiogenic theranostics.

9.6. Carbon based nanomaterials

Carbon as the second most abundant element in the human body attracted a lot of attention in nanomedicine. Recently, carbon based nanomaterials (nanodiamonds, carbon nanodots, carbon nanotubes, graphene, fullerenes, carbon nanofibers, carbon nanocone-disks and nanohorns) have become

important materials for potential biomedical applications due to their unique chemical and physical properties (*i.e.* optical, thermal, electrical, mechanical *etc.*).[164,165] Various carbon based nanoparticles and their various allotropes exhibited profound anti-angiogenic activities, observed by various *in vitro* and *in vivo* assays. Grodzik *et al.* reported the anti-angiogenic properties of ultradispersed detonation diamond (UDD) nanoparticles towards a glioblastoma multiforme (GBM) tumor model developed on a chorioallantoic membrane.[124] Murugesan *et al.* reported the anti-angiogenic activities of various carbon materials (graphite, nanotubes, multiwalled carbon and fullerenes) towards a CAM model.[166] The nanomaterials significantly reduce the tumor volume, weight and vessel area associated with down-regulation of VEGF and b-FGF2-induced angiogenesis. Wierzbicki *et al.* investigated the angiogenic activities of different carbon nanomaterials (diamond nanoparticles, graphite nanoparticles, graphene nanosheets, multi-wall nanotubes and C60 fullerenes) on blood vessel development evaluated in an *in ovo* chick embryo chorioallantoic membrane model. Among those nanomaterials, diamond nanoparticles and multi-walled nanotubes showed the maximum anti-angiogenic properties. Surprisingly, fullerene exhibited the opposite effect, pro-angiogenic activity. Graphite nanoparticles and graphene had no effect on angiogenesis. Diamond nanoparticles reduced the expression of VEGF-R.[131] Fig. 6 shows the thickness of CAM tissue cross sections by the treatment of different carbon based nanomaterials. Diamond nanoparticles and MWNT exhibited 2–3 fold decrease in CAM tissue thickness, indicating high anti-angiogenic activities of those nanomaterials. Molecular studies showed that the enhanced anti-angiogenic activities of these nanomaterials were associated with the

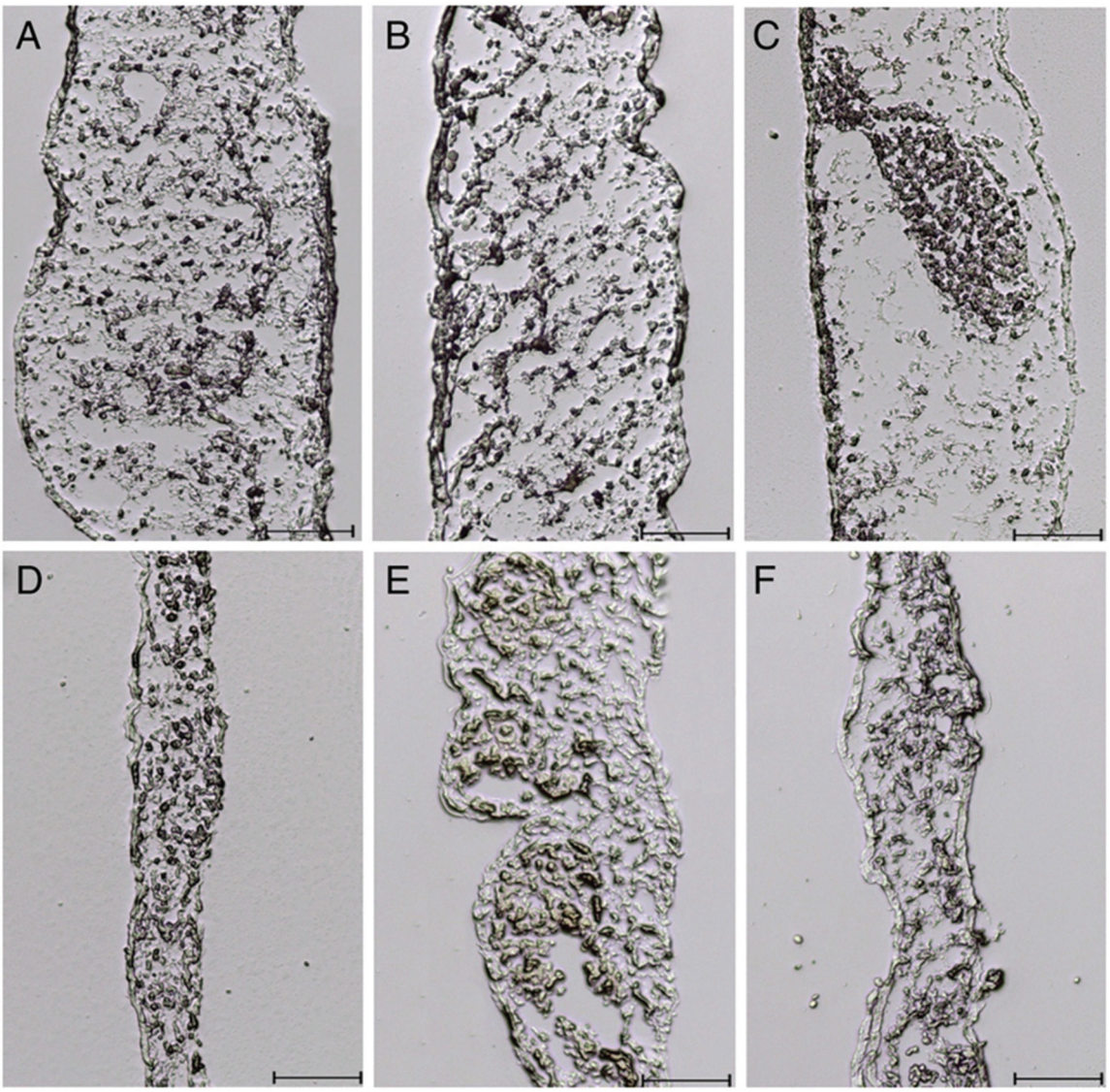

Fig. 6 Cross sections of CAM tissue treated with carbon nanoparticles. (A) Control, (B) GNS, (C) NG, (D) ND, (E) C60 and (F) MWNT. Scale bar, 100 μm. Reprinted with permission from ref. 131. Copyright (2013) Springer.

down-regulation of KDR, which decreases the hypoxia mediated angiogenesis.[131]

Reactive oxygen species (ROS) can play a crucial role in cellular machinery. It is well established that lower concentrations of ROS can activate the endothelial cell proliferation, migration, and tube formation whereas higher concentration of ROS may kill the cells. Graphene and graphene oxide show cytotoxicity due to uncontrolled formation of ROS, reported by several research groups.[167] ROS play a crucial role in modulating the angiogenic activity.[108,109] Recently, our group showed the ROS dependent switchover of angiogenesis and anti-angiogenesis of both graphene oxides (GO) and reduced graphene oxides (rGO), investigated by various *in vitro* and *in vivo* assays.[137] High concentrations of both GO and rGO (>100 ng mL^{-1}) exhibited inhibition of endothelial cell proliferation, migration and tube formation due to the generation of excessive ROS that can trigger the down-regulation of pAKT and peNOS, leading to anti-angiogenesis (Fig. 7). Similarly, low concentrations of GO and rGO produce low ROS that help in angiogenesis.

Chaudhuri *et al*. demonstrated the anti-angiogenic activity of fullerenols or doxorubicin-conjugated fullerenols in embryonic zebrafish and murine melanoma tumor angiogenesis models in C57/BL6 mice.[113] Fig. 8 shows that both fullerenols and doxorubicin-conjugated fullerenols inhibited the sprouting of neovascularization observed by alkaline phosphate staining upon treatment of these nanomaterials in the yolk sac next to the subintestinal vessels of 48 hpf embryonic zebrafish for 48 hours. Again, they found that both fullerenols and doxorubicin-conjugated fullerenols exhibited excellent anti-tumor activities in a murine melanoma model accompanied by a decreased amount of blood vessel density observed by immunostaining (Fig. 9).[113] These results suggest the potential therapeutic application of fullerenols as anti-angiogenic agents as well as next generation cancer drug delivery vehicles.

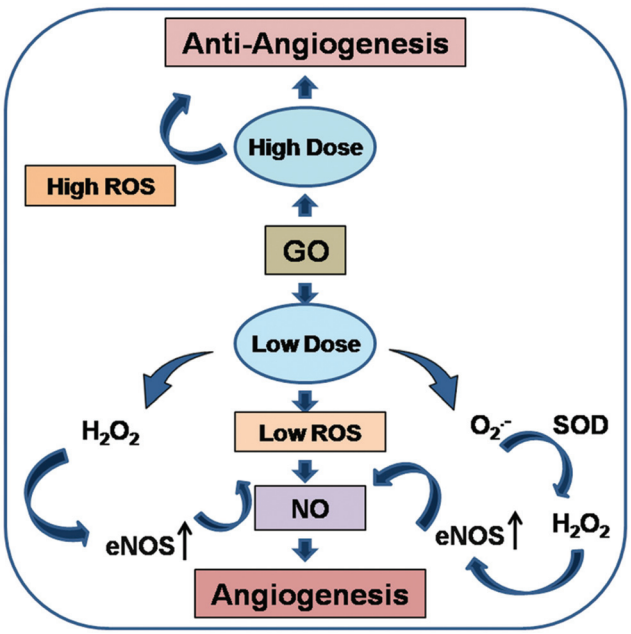

Fig. 7 Plausible mechanism of dose-dependent switchable angiogenesis of graphene oxide (GO) through ROS formation and NO signaling. Reprinted with permission from ref. 114. Copyright (2015) Wiley-VCH.

Meng *et al.* reported the anti-angiogenic activity of multiple hydroxyl group functionalized (Gd@C82(OH)22) fullerenic nanoparticles (f-NPs) in *in vitro* and *in vivo* breast cancer models.[121] *In vitro* analysis of mRNA and protein levels confirmed that f-NPs inhibited more than 10 fold angiogenic factors (Cxcl1, Cxcl2, Cxcl5; Fgf1, Fgf6, Fgfr3; Mmp19, Mmp2 and Mmp9; Lama5, Tgfb1, Tgfb2 and Lama5) in mRNA level, further confirmed by western blot analysis. Dose dependent inhibition of *in vitro* cell viability and migration ability of human microvascular endothelial cells confirms the anti-angiogenic activity of f-NPs. Further, authors investigated the *in vivo* anti-tumor potential of f-NPs (3.8 mg kg^{-1}) in a breast cancer model. The enhanced anti-tumor efficacies compared to the standard drug paclitaxel were associated with the decrease in tumor blood vessels, tumor weight along with the decrease in tumor microvessel density (>40%) (Fig. 10). Also, f-NP treated tumor tissue showed less blood perfusion (>40%), *i.e.* the speed of blood supply to tumor tissues, than control tumor tissue, observed by MRI imaging, supporting the enhanced anti-angiogenic activity of f-NPs. Finally, the TEM picture shows the damaged tumor vessel integrity in f-NP treated tumors whereas the control tumor did not affect the normal blood vessels in kidney tissues, further supporting the anti-angiogenic activity of f-NPs (Fig. 11).

9.7. Chitosan nanoparticles

Chitosan is an interesting polymer that was extensively used in the field of biomedical applications because of its biocompatibility, non-toxicity, biodegradability, antimicrobial activity and low immunogenicity. Jayakumar nicely reviewed the biomedical applications of chitin and chitosan based nanomaterials.[168] Xu *et al.* reported that chitosan nanoparticles (CNPs) inhibit the development of human hepatocellular carcinoma (HCC) xenografts through an anti-angiogenic mechanism.[120] The researchers investigated the effect of CNPs on tumor growth using a model of nude mice xenografted with human HCC (BEL-7402) cells. They observed that CNPs considerably inhibited tumor growth and induced tumor necrosis in a dose and time dependent manner. H&E staining of chitosan treated tumor sections showed dramatically increased amount of necrotic area in those tissue sections. Mechanistic studies involving immunohistochemistry and q-RT-PCR analysis revealed that the anti-tumor effects of chitosan nanoparticles were due to the anti-angiogenic effect associated with the impaired levels of VEGF and VEGFR-2. Thus, the suppression of VEGFR-2 leads to the blockage of VEGF, and exhibits anti-angiogenic activity towards endothelial cell proliferation. Because of low toxicity, CNPs and its derivatives may be used as potent anti-cancer drugs. Additionally, chitosan nanoparticles were used as delivery vehicles for the delivery of several anti-cancer drugs and siRNA for the treatment of

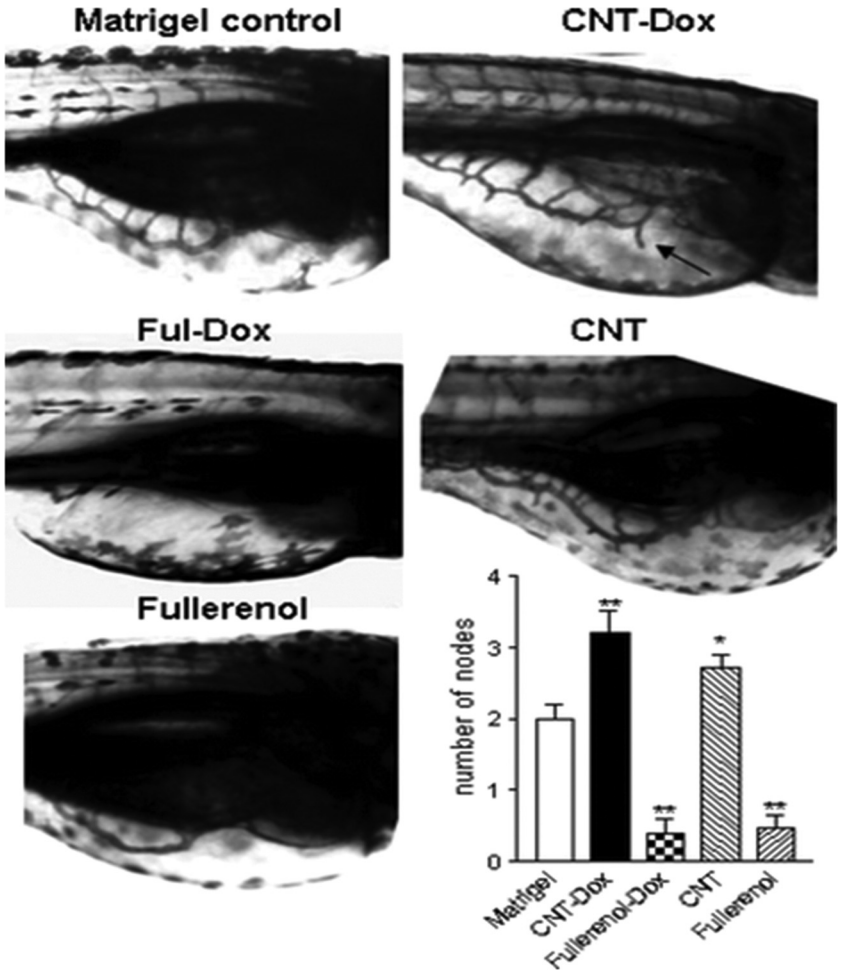

Fig. 8 Effect of nanoparticle–doxorubicin conjugates on angiogenesis in embryonic zebrafish. The nanoparticles were injected in the yolk sac near the subintestinal vessels (SIV) of 48 hpf embryonic zebrafish and incubated for a further 48 h. Images were taken after alkaline phosphatase staining in order to visualize the blood vessels. The arrow indicates the sprouting of neovasculature from SIV. Graphs show the morphometric quantification of the effects on the number of nodes. Data shown are the mean ± SE (n = 4–6). *p < 0.01 *vs.* Matrigel alone control (ANOVA followed by the Newman Keuls *post hoc* test). Reprinted with permission from ref. 113. Copyright (2010) American Chemical Society.

cancers. For example, Pillé *et al.* reported the administration of chitosan-coated polyisohexylcyanoacrylate nanoparticle containing anti-RhoA siRNA, which showed inhibition of tumor growth and angiogenesis in an aggressive breast cancer mouse xenograft model.[169]

9.8. Tetrac nanoparticles

Tetrac (tetraiodothyroacetic acid) is a deaminated analogue of L-thyroxine (T(4)) that inhibits the pro-angiogenic activities of T(4) and 3,5,3′-triiodo-L-thyronine, other cell surface based growth factors for thyroid hormone on integrin αvβ3 receptor, and ultimately induces apoptosis and anti-cancer activities.[122] Yalcin *et al.* reported that tetrac nanoparticles exhibit the anti-angiogenic effect along with inhibition of tumor growth in renal cell carcinoma xenografts.[122] Tetrac is a well established blocking agent of L-thyroxine as well as other cell surface based angiogenic growth factors on integrin αvβ3, which are expressed both in cancer and in vascular endothelial cells.[122] In that work, they showed the anti-angiogenic as well as tumor inhibitory effects of tetrac nanoparticles by tumor cell implants in a CAM model and a renal xenograft model in nude mice. 1.86 mg kg^{-1} dose of tetrac nanoparticles exhibited excellent anti-tumor efficacy in tumor xenograft as well as inhibition of tumor growth and tumor angiogenesis in a CAM model. Their findings indicate that anti-angiogenic and anti-tumor activities of Tetrac and Tetrac NP could be useful for the treatment of cancers.

9.9. Peptide conjugated nanoparticles

Bartczak *et al.* demonstrated the inhibition of *in vitro* angiogenesis using functional peptide coated gold nanoparticles.[127] The oligo-ethylene glycol capped gold nanospheres were incubated with a peptide that selectively interacts with receptors of cells, leading to the inhibition of angiogenesis without causing toxicity. The anti-angiogenic activity was investigated by several *in vitro* assays. P3-peptide conjugated AuNPs showed

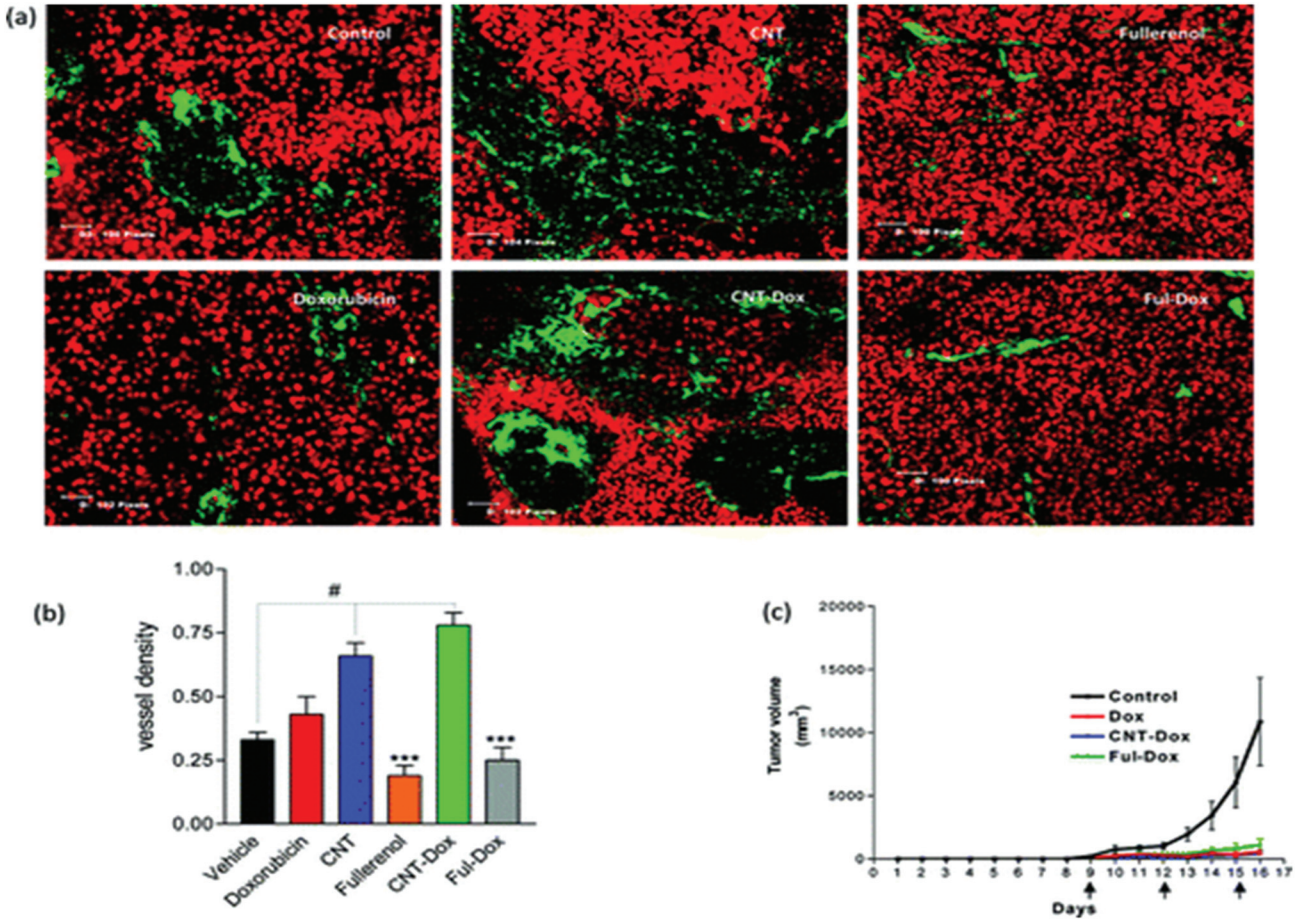

Fig. 9 Effect of nanoparticle–doxorubicin conjugates on tumor angiogenesis. B16/F10 melanoma cells were implanted subcutaneously in the flanks of C57/BL/6 mice. Each group received three doses of the appropriate treatment every third day. (a) Tumor cryosections were immunolabeled with a von Willebrand Factor (vWF) antibody and then probed with an Alexa 488 conjugated secondary antibody. The sections were counterstained with propidium iodide. Images were captured with a Nikon Eclipse T*i* fluorescence microscope using QCapturePro software. Magnification = 10×. Scale bar is shown in pixels. One pixel = 0.45 μm. (b) Graph shows the morphometric quantification of vessels using the ratio of green pixels (Alexa 488)/red pixels (PI). Data represent the mean ± SE of $n \geq 6$: (#) $p < 0.05$, (***) $p < 0.01$ (ANOVA followed by the Newman Keuls *post hoc* test). (c) Effect of treatment on tumor growth. Arrows indicate the days of injection. Reprinted with permission from ref. 113. Copyright (2010) American Chemical Society.

anti-angiogenesis due to higher concentration of ROS that blocks the capillary formation in endothelial cells compared to P1- and P2-conjugated AuNPs. Mechanistic studies showed that the relative levels of several anti-angiogenic cytokines (pentraxin, PF4, GMCSF, Coag FIII, prolactin, endostatin *etc*.) and pro-angiogenic factors (PDGF, VEGF, IGFBP-2 *etc*.) either increased or decreased from the normal levels. Also, the authors explained that the engineered P3 peptide could bind to the NRP-1 receptor on the endothelial cell surface for targeted receptor based internalization. Anti-angiogenic and anti-glioma therapies using EG-PLA nanoparticles modified with the APTEDB peptide were demonstrated by Gu *et al.*[170] Huang *et al.* demonstrated that the tumor-targeting and micro-environment-responsive smart nanoparticles could be useful for cancer therapy using combination therapy of anti-angiogenesis and apoptosis.[118] Some investigators utilized chitosan–dextran sulfate nanoparticles for the delivery of anti-angiogenesis peptides for cancer therapy.[171]

9.10. Perfluorocarbon nanoparticles

A recent report demonstrates the clinical applications of perfluorocarbon nanoparticles in targeted therapy and molecular imaging.[172] Caruthers *et al.* developed perfluorocarbon nanoparticles that could be utilized for $\alpha v\beta 3$ targeted anti-angiogenic drug delivery for cancer, atherosclerosis and other diseases.[134,139]

10. Plausible mechanism for nanoparticle based anti-angiogenesis

Several groups proposed various mechanisms for the anti-angiogenic activity of nanoparticles or nanoparticle based drug delivery systems in cancer therapy. The main mechanistic framework of anti-angiogenesis involves the attachment or binding of nanoparticles with the VEGF that prohibits the VEGF from attaching with VEGFR, and results in the down-

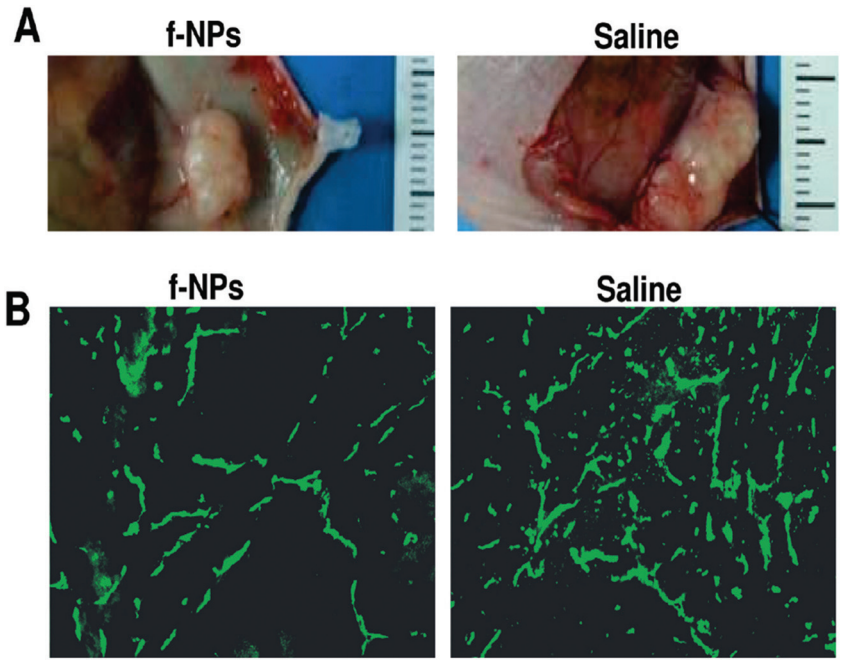

Fig. 10 The *in vivo* anti-angiogenesis effects of the f-NPs in mice. (A) Morphology of tumor tissue with the f-NPs or saline treatment. Less visible blood vessels can be found in the f-NP group. (B) The tumor tissues were stained for CD31 by immunohistochemistry. The f-NP treatment significantly reduced the microvessel density (MVD) in tumor tissue compared to that of the control. Reprinted with permission from ref. 121. Copyright (2010) American Chemical Society.

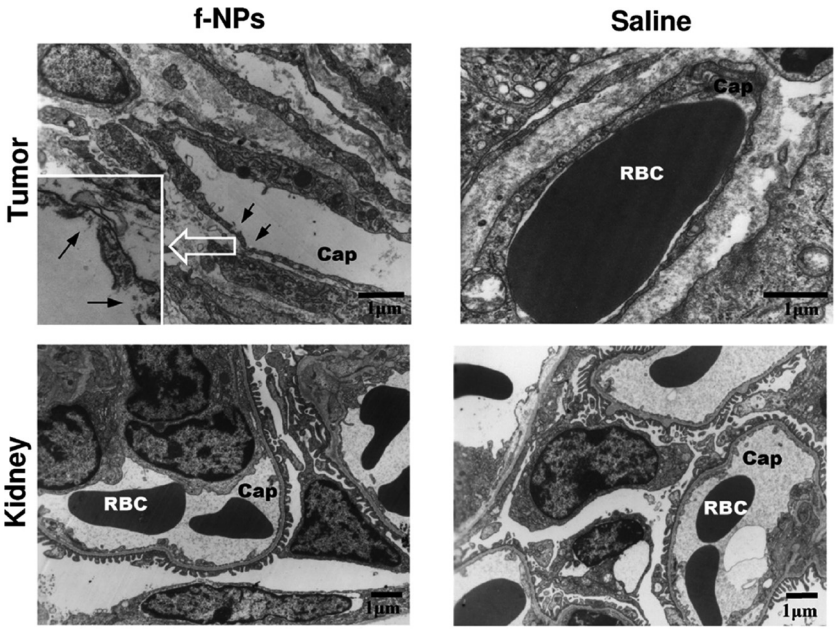

Fig. 11 Electron microscopy to determine the ultrastructural changes in tumor blood vessels and normal blood vessels. The f-NPs further damaged the integrity of tumor vessels, but had limited effects on normal blood vessels in kidneys. "Cap" indicates the capillary vessel, and "RBC" denotes red blood cells. Reprinted with permission from ref. 121. Copyright (2010) American Chemical Society.

regulation of VEGFR, mainly responsible for the inhibition of angiogenesis. VEGFR consists of VEGFR-1, VEGFR-2 and VEGFR-3, which bind to any one of VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E to activate the downstream pathways, mainly MAPK signalling pathways, AKT signalling pathways, and JNK/c-Jun pathways, which trigger the vasculogenesis or angiogenesis. VEGF-A has two major isoforms: the heparin binding growth factor (VEGF165) and the non-heparin

binding growth factor (VEGF121) that are the most vital pro-angiogenic factors during angiogenesis. Down-regulation of either VEGF or VEGFR can lead to the down-regulation of these downstream pathways, resulting in anti-angiogenesis.[119,120,125,128,132,137,149] Fig. 12 shows that down-regulation of VEGFR2 expression by copper oxide nanoparticles might suppress several VEGFR2 mediated downstream pathways' activation, thus inhibiting angiogenesis.[130] On the other hand, down-regulation of VEGF receptor or kinase insert domain receptor (KDR) can decrease the hypoxia-mediated angiogenesis *via* the down-regulation of HIF-1, consequently inhibiting the VEGF mediated angiogenesis.[131] Apart from these, nanoparticles can inhibit the functional activity of various growth factors including vascular endothelial growth factor 165 (VEGF165), heparin growth factor (HGF), heparin-binding EGF-like growth factor (HB-EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), stromal cell-derived factor 1 (SDF1), platelet-derived growth factor (PDGF) *etc*.[127] Inhibition of these growth factors can lead to anti-angiogenesis. Mukherjee *et al.* demonstrated that gold nanoparticles bound to VEGF165 (heparin binding growth factor) through the heparin binding domain and inhibited the activity of KDR and triggered the anti-angiogenesis cascade. Again, the same group showed that bare AuNPs bind to basic fibroblast growth factor which is another crucial cell mitogen and mediator of angiogenesis, thus inhibiting the fibroblast cell proliferation along with VEGF-induced permeability and angiogenesis towards *in vivo*.[137] Furthermore, several groups demonstrated that different nanoparticles can alter the level of various anti-angiogenic cytokines (pentraxin, PF4, GMCSF, Coag FIII, prolactin, endostatin *etc*.) and pro-angiogenic factors (PDGF, VEGF, IGFBP-2 *etc*.) from normal levels.[127] ROS also play a very crucial role in maintaining the angiogenic balance inside the body. It is well established that excessive formation of ROS can show potential toxicity while control production of ROS can help in endothelial cell proliferation, *i.e.* angiogenesis. However, excessive production of ROS can lead to cell death. Our group showed that graphene oxide (GO) and reduced graphene oxide (rGO) exhibited a switchover role between angiogenesis and anti-angiogenesis based on the concentration of treatments, which control the production of ROS.[137] The intracellular formation of control ROS in <100 ng mL^{-1} doses of GO and rGO and reactive nitrogen species as well as the activation of phospho-eNOS (p-eNOS) and phospho-Akt (p-AKT) might be the plausible reason for angiogenesis,[137] whereas at higher doses (>100 ng mL^{-1}), both GO and rGO caused excessive ROS production, resulting in anti-angiogenesis. Anti-angiogenic activity of gold nanoparticles occurred due to the

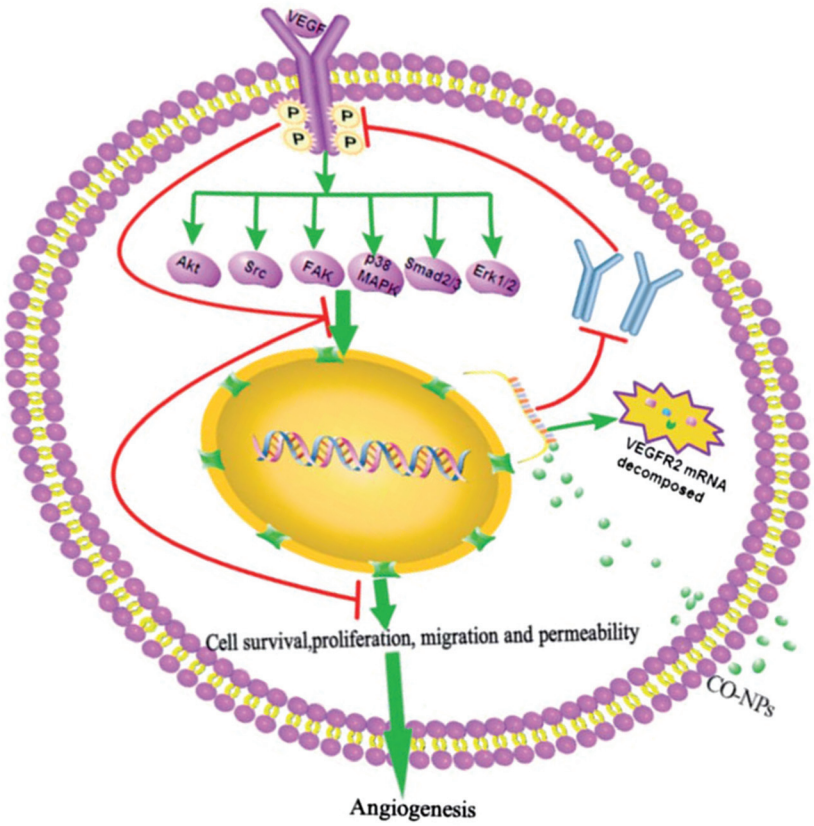

Fig. 12 CO-NPs inhibit angiogenesis *via* down-regulation of VEGFR2 expression. VEGFR2 is a key regulator of angiogenesis through activating downstream pathways. CO-NPs were able to suppress VEGFR2 expression both at mRNA and protein levels, thus inhibiting VEGFR2 mediated angiogenesis. Reprinted with permission from ref. 130. Copyright (2014) The Royal Society of Chemistry.

inhibition of VEGF165-induced migration and tube formation of endothelial cells *via* the Akt pathway.[126] Xu *et al.* demonstrated the anti-angiogenic activity of chitosan nanoparticles to human hepatocellular carcinoma xenografts.[120] Several critical factors (size, shape, surface and charge of nanoparticles, functional group on the surface, dissolution of particles, release of metal ion from nanomaterials/nanoconjugates, UV light, aggregation, interaction of nanoparticles with cells or cell surface, inflammation, and pH of the medium) can affect the generation of ROS, responsible for anti-angiogenic activity.[167] Thus, the mechanism of anti-angiogenesis of compounds or nanomaterials depends on various factors, often inter-related, and needs to be investigated thoroughly to find out new therapeutic targets.

11. Future opportunities, challenges and directions

Nanomaterials were extensively used in biological and medicinal applications (targeted drug/gene/antigen/siRNA/shRNA delivery, immunoassays, clinical chemistry genomics, biosensorics, photothermolysis of cancer cells and tumors, optical bioimaging *etc.*) due to their exceptional physico-chemical properties. However, nanomaterials or any foreign material often exhibits potential toxicity in different parts of the human body. Thus, it is very important to perform systematic biosafety, efficacy, metabolic long-term fate (*in vitro* and *in vivo*), interaction of the particles with immune cells, potential long-term toxicity, and pharmacokinetic studies in an animal model before using the nanomaterials in clinical trials.

The future challenge is to synthesize and develop novel anti-angiogenic nanomaterials that should specifically target the cancerous cells, without showing any toxicity in normal organs. Again, in the combinatorial approach, these anti-angiogenic nanomaterials conjugated with already established FDA approved chemotherapeutic drugs (anti-angiogenic drugs) could help develop better and effective therapeutic strategies. Several issues should be carefully investigated before clinical trials; for example: (a) biocompatibility of the nanomaterials, (b) biodegradability and secretory pathways of these nanomaterials from the body, (c) the best route of administration and the number of doses in a certain time and (d) the use of a combinatorial approach (anti-angiogenic nanomaterials associated with anti-cancer drugs) to reduce the systematic toxicity and increase the therapeutic efficacy. Critical information including uptake, retention, and clearance of these nanoparticles should be carefully studied.[141]

11.1. Challenges and difficulties of production

Generally, production of nanoparticles and nanoconjugates on an industrial scale poses several challenges and obstacles in scale up of nanomedicine. The laboratory scale top-down or bottom-up approaches to synthesize nanoparticles vary severely from commercial manufacturing. This may require the use of organic solvents, high temperature reactors, sonication, milling, high-speed homogenization, emulsification, evaporation of organic solvents, crosslinking, filtration, centrifugation, or lyophilization.[173] During the early stage of development of nanomaterials at the lab scale, researchers should consider a suitable approach that may be useful for large-scale manufacturing purposes. Also, it is very crucial to identify the synthesis conditions that can alter the yield, quality and effectiveness of the nanomedicine products. These include the ratio of reactants, amount of drugs or targeting moieties to be used, the type of solvent, and stabilizer/crosslinker, the oil-to-water phase ratio, and the mixing conditions, reaction time, temperature, addition rate, pressure, pH *etc*. A little change in any of these reaction conditions can generate biologically inactive, unstable and undesired products with a high amount of impurity. Thus, it is critical that the synthesis and formulation processes of nanomedicine products must be robust with high reproducibility, well characterized and tested before commercial large scale manufacturing.

11.2. Targeting

In general, nanoparticles can target leaky tumor vasculature by the enhanced permeability and retention (EPR) effect (passive targeting). Hence, the non-specificity towards healthy cells and tissues is still high. To overcome this non-specific toxicity of nanoparticles toward healthy organs and tissues, several targeting agents were employed to enhance the tumor specific uptake and activity (active targeting). For obtaining enhanced therapeutic efficacy and decreased nonspecific toxicity of anti-angiogenic drugs, the use of active targeting agents (*e.g.*, mAbs, $\alpha_v\beta_3$ integrin antibody, antiangiogenic peptides) has become a successful alternative method.[63,117,174,175] Integrins are a family of cell surface receptors. Researchers found that $\alpha_v\beta_3$ integrin was dramatically upregulated during neovascularization in most of the cancer settings.[176] Consequently, targeting of integrin receptor ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) by either $\alpha_v\beta_3$ integrin antibody or anti-angiogenic peptides (RGD/NGR) did show some exciting therapeutic results.[177–179] Several published reports demonstrated the excellent targeting capabilities with enhanced therapeutic efficacy and negligible side effects of these targeting agents when applied with the combination of nanoparticles.[6,180–183] Thus active targeting can be an alternative strategy to overcome the nonspecificity and toxicity of anti-angiogenic nanoparticles.

11.3. The diffusion

Diffusion and penetration of nanoparticles through cell and tissue barriers pose a crucial challenge for the uptake and efficacy of nanomedicine. For intravascular delivery of nanoparticles, the main barriers which play an important role are (i) initial immune rejection or clearance in the liver and spleen, (ii) permeation across the endothelium into target sites, (iii) penetration through the interstitial tissue, (iv) endocytosis or receptor mediated entry in target cells, (v) diffusion through the cytoplasm and release of a therapeutic moiety and (vi) possible entry into the nucleus, if necessary.[184] Other alternative routes including skin and mucosal membranes of

the nose, intestine lungs, and vagina pose a significant barrier to delivery through diffusive resistance of these tissues. Recently, Cho *et al.* demonstrated that the cellular uptake and penetration of AuNPs depend on the diffusion and sedimentation velocities of the nanoparticles and are independent of their size, shape, morphology, surface coating, density, and initial concentration.[185] They showed that nanoparticles with faster sedimentation rates exhibited greater differences in uptake in the upright configuration than the inverted one.

11.4. Toxicological and immunological aspects

Toxicity of different nanomaterials should be taken into account before clinical implications. Understanding human health risk and toxicity associated with the rapidly emerging different nanomaterials poses an enormous challenge due to the wide range of applications accompanied by the different routes of exposure of these materials. Several groups reported the *in vivo* toxicity (acute and chronic) of nanomaterials including copper oxide, silver, platinum, zinc oxide, cerium oxide *etc*.[186,187] Also, a few groups including our group demonstrated the non-toxic nature of various nanomaterials (AuNPs, EHNs *etc*.) in animal models.[188–190] There are numerous published reports that demonstrate the detailed investigation of absorption, bio-distribution, excretion/clearance and toxicity profile of mesoporous silica materials in *in vivo* models.[191,192] Liu *et al.* demonstrated the low toxicity (based on mortality, histopathological examination, hematological study, clinical features, and blood biochemical studies) of mesoporous silica nanoparticles after intravenous (IV) injection (20–80 mg kg^{-1}) at a single dose or repeated administrations in mouse models.[193] They also investigated the bio-distribution and accumulation of these nanoparticles (mostly found in the liver and spleen) and clearance from the body. Similarly, gold nanoparticles and EHNs were found to be nontoxic when analyzed by blood biochemical studies, serum clinical chemistry, histopathology and other studies.[188,189,194] On the other hand, platinum, cerium oxide, zinc nanoparticles were found to have nephrotoxicity, liver toxicity, acute and chronic toxicity when analyzed by several assays.[186,187,195] Therefore, it is urgently needed to carefully evaluate the detailed toxicity studies by considering various parameters that include serum and blood parameters, tissue histopathology, genotoxicity, pharmacokinetics, pharmaco-dynamics, and immunological responses. A recent review article by Hansen *et al.* discussed about 400 studies with 965 nanomaterials that addressed the cytotoxicity, the mammalian toxicity, and the ecotoxicity of the different materials.[196] Because of the diversity of nanomaterials, it is really difficult to link specific properties of nanomaterials with the tolerances in biological systems.

11.5. Biodegradability and clearance

Another important issue for any nanomaterials is bio-degradability. It is well established that polymer nanoparticles, micelles, and liposomes are biodegradable in nature and easily cleared from the body easily within a very short time span. However, metal based nanomaterials might be bio-degradable with a slow metabolic degradation process.[188,197,198] A few recent studies suggested that metal nanoparticles are slowly excreted through feces and urine,[188,190] although the detailed mechanism behind the clearance of metal nanoparticles through excretory and metabolic pathways is poorly understood. Long-term deposition of metallic substance in body organs can generate enduring toxic effects.

In another report, Rengan *et al.* demonstrated the biodegradability nature of liposomal gold nanoparticles (Lipos-AuNPs), an efficient drug delivery system for photothermal cancer therapy.

The delivery system underwent metabolic degradation in the liver and hepatocytes. The particles can be easily excreted by the renal route along with the hepato-biliary route.[188] Also, *in vivo* bio-distribution of the liver, kidney as well as blood plasma showed a gradual decrease in the amount of deposited gold with time, confirmed by ICP-MS analysis. Feces and urine samples up to 14 days detect significant amounts of gold, confirming the slow excretion process from the body. It was also discussed that positively charged gold nanoparticles may overcome possible charge repulsion by the negatively charged glomerular basement membrane (GBM) present in the nephrons and entered renal excretion through urine. Cassano *et al.* recently showed the complete degradation of silica nanospheres containing gold nanoparticles (AuSi) in full serum within a few hours allowing renal clearance, thus overcoming the tissue deposition of nanoparticles.[197] The mechanism of clearance indicated that silica nanospheres were subjected to degradation into soluble silicic acid and excreted through the renal pathway. Also, authors proposed that the remaining AuNPs may coated by endogenous glutathione completely followed by renal clearance. Park *et al.* proposed the possible biodegradation of luminescent silica nanoparticles (LPSiNPs) into soluble silicic acid and cleared through the renal pathway without any toxic adverse effect by self-destruction.[198] Our group demonstrated the excretion of EHNs through feces in 24 hours of post treatment suggesting the possible removal of EHNs from the body.[190] However, the study to find out the reasons for clearance of the nanorods from the body is under investigation. Kurapati *et al.* recently reported that graphene oxides completely biodegrade and metabolize in the presence of a human enzyme, *i.e.* myeloperoxidase (hMPO) derived from human neutrophils containing a low concentration of hydrogen peroxide.[199] The extent of degradation by enzymes depends on the colloidal stability of the nanomaterials, which is a key aspect of its breakdown. Choi and co-workers demonstrated that quantum dots nanoparticles with less than 5.5 nm size were rapidly and efficiently excreted and eliminated through the urinary route from the body in rodents.[200] All of these detailed studies suggest that size, shape and morphology can dictate the bio-distribution, transport, kinetics, accumulation, clearance, fate, and subsequent molecular effects of the nanomaterials in a living organism, which is more complex in *in vivo* systems, making this an active area of modern research.

The advancement of nanotechnologies with rapidly growing industry (~$1 trillion by 2015) might expect the possible

solution for cancer cure after taking considerable step towards general safety, environmental effects, and potential health effects.[201,202] Currently, researchers are investigating the development of novel anti-angiogenic nanomaterials for the treatment of cancers and the relationship with tumor growth and survival in order to establish a new therapeutic approach.

12. Conclusions

Most of the effective anti-angiogenic drug mediated cancer therapies are limited by the unavoidable progress of drug resistance. Over the last decade, nanotechnology has been used for multifunctional activities in biology and medicine. Anti-angiogenic nanomaterials are likely to revolutionize the face of medicine in the next decade towards cancer therapy. Anti-angiogenic nanomaterials can be delivered solely or probably in combination with additional anti-cancer drugs/siRNA/peptides depending on the stages and advancements. The safety of anti-angiogenic treatment requires special attention, and optimization of the dose and duration of the nanomaterials also needs to be evaluated. All the results taken together, this review article highlights the anti-angiogenic properties of recently developed anti-angiogenic nanomaterials and their potential applications in cancer treatment. Finally, various factors including bio-safety, efficacy, metabolic long-term fate (*in vitro* and *in vivo*), interactions of the particles with immune cells, potential long-term toxicity study, and pharmacokinetic study in an animal model should be thoroughly examined before using these novel anti-angiogenic nanomaterials in clinical trials. The nanomedicine approach allows researchers to develop novel nano-engineered anti-angiogenic nanomaterials that could be the most promising and feasible alternative technologies for cancer therapy in the near future. The application of angiogenesis inhibitors using the nanomedicine approach could be useful as a new promising treatment strategy for cancer research.

Abbreviations

AFM	Atomic force microscopy
AgNPs	Silver nanoparticles
Ang	Angiopoietin
AuNPs	Gold nanoparticles
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
BMDCs	Bone marrow-derived cells
BRECs	Bovine retinal endothelial cells
CAM	Chick chorioallantoic membrane
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
CML	Chronic myelogenous leukemia
CNPs	Carbon nanoparticles
COX	Cyclooxygenase
CSC	Cancer stem cells
CuNPs	Copper nanoparticles
CXCL	The chemokine (C-X-C motif) ligand
DLA	Dalton's lymphoma ascites
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ECs	Endothelial cells
EHNs	Europium hydroxide nanorods
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinases
EPCs	Endothelial progenitor cells
EPR	Enhanced permeability and retention
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiforme
G-CSF	Granulocyte colony stimulating factor
GIST	Gastrointestinal stromal tumor
GMCSF	Granulocyte macrophage colony-stimulating factor
GNS	Graphene nanosheets
GO	Graphene oxide
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor/heparin growth factor
HIF-1	Hypoxia-inducible factor 1
HUVEC	Human umbilical vein endothelial cell
ICP-MS	Inductively coupled plasma mass spectroscopy
IFN-α, -β and -γ	Interferon-α, -β and -γ
IGF	Insulin like growth factor
IL-1, -4, -12, -18	Interleukin-1, -4, -12, -18
KDR	Kinase insert domain receptor
Lama-5	Laminin alpha 5
LPSiNPs	Luminescent silica nanoparticles
MAPK	Mitogen-activated protein kinases
MMP-2	Matrix metalloproteinase-2
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MWNT	Multi-walled nanotubes
NCe	Nanoceria
ND	Nanodiamonds
NG	Nanographite
NGR	Asparagine-glycine-arginine
NPs	Nanoparticles
NRP1	Neuropilin 1
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDT	Photodynamic therapy
PEDF	Pigment epithelium-derived factor
PEG	Polyethyleneglycol
PEI	Polyethyleneimine
PF4	Platelet factor 4
PRECs	Porcine retinal endothelial cells
RBCCs	Recruited bone marrow-derived circulating cells
RGD	Arginyl glycyl aspartic acid

rGO	Reduced graphene oxide
ROS	Reactive oxygen species
SDF1	Stromal cell-derived factor 1
shRNA	Small hairpin ribonucleic acid
SiNPs	Silicate nanoparticles
siRNA	Small interfering ribonucleic acid
TAF	Tumor angiogenesis factor
TEM	Transmission electron microscopy
Tetrac	Tetraiodothyroacetic acid
TKI	Tyrosine kinase inhibitors
VDA	Vascular disrupting agents
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

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