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Application of gold nanoparticles for gastrointestinal cancer theranostics: a systematic review

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Abstract

Gold nanoparticles (GNPs) are readily synthesised structures that absorb light strongly to generate thermal energy which induces photothermal destruction of malignant tissue. This review examines the efficacy, potential challenges and toxicity from *in vitro* and *in vivo* applications of GNPs in oesophageal, gastric and colon cancers. A systematic literature search of Medline, Embase, Web of Science and Cochrane databases was performed using PRISMA guidelines. Two hundred and eighty-four papers were reviewed with sixteen studies meeting the inclusion criteria. The application of GNPs in eleven *in vivo* rodent studies with GI adenocarcinoma demonstrated excellent therapeutic outcomes but poor corroboration in terms of the cancer cells used, photothermal irradiation regimes, fluorophores and types of nanoparticles. There is compelling evidence of the translational potential of GNPs to be complimentary to surgery and feasible in the photothermal therapy of GI cancer but reproducibility and standardisation require development prior to GI cancer clinical trials.
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Key words: Gold nanoparticles; Gastrointestinal cancer; Photothermal therapy; Oesophageal; Gastric; Colon

In 2010, an average of 430 people died from cancer daily in the United Kingdom, which equates to one person every four minutes. 1 in 2 people in the UK will develop cancer in their lifetime,[1] whilst in the United States, 1 in 3 women and 1 in 2 men will develop cancer. In some nations, cancer will surpass heart disease as the commonest cause of mortality.[2] The incidence of gastrointestinal (GI) cancers is increasing since the mid-1970s in the UK, and primarily includes oesophageal, gastric and colorectal carcinomas, with a Western preponderance towards adenocarcinomas. Colorectal and oesophageal cancers are now the 4th and 8th commonest cancers worldwide respectively.[3] These cancers are often being detected rather late in their course, as their detection relies heavily on symptomatic reporting and on non-specific screening methods.[4] The 5-year survival rates of patients who are deemed suitable for definitive treatment range from 5 to 20% for oesophageal cancer, 10-15% for proximal gastric cancer and 6-75% for colorectal cancer.[3,5,6]

Generally the first-line treatment of solid and established gastrointestinal tumours in the UK is neoadjuvant chemo(radio)therapy, followed by surgical excision and depending on the grade/stage of the tumour, adjuvant chemotherapy. Single modality treatment is largely ineffective. Chemotherapy has a substantial failure and intolerance rate due to inadequate localisation of drugs to cancer-specific tissues and systemic side effects.[7,8] Radiation, on the other hand is unable to eliminate all loco-regional recurrences and cure localised cancers due to the inherent resistance of some cancer cells towards ionising radiation.[9] Neither radiotherapy nor chemotherapy has shown significant survival benefit,[10] and the results from surgery as a sole entity are meagre without the summative complementary effects from chemo-radiotherapy. The need for establishing personalised medicine as a means of providing tailor-made targeted delivery of therapy for specific cancers to individual patients seems increasingly essential.

Conflicts of interest: none.

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The Nobel Prize winner Richard Feynman first proposed nanotechnology in 1959.[11] "Nano" is Greek for "dwarf" and nanotechnology comprises particles that are of the order of 1 billionth of a metre (10^{-9} m). The National Nanotechnology Initiative (NNI) defines nanotechnology at dimensions of roughly 1-100 nanometres (nm).[12] By this definition, the largest nanoparticle (NP) is approximately six to eight hundred times smaller than the width of a strand of hair and approximately 100-10,000 times smaller than a human cell. The past 25 years has seen an intensified interest in nanotechnology, with the development of a multitude of different shaped NPs for material science and nanomedicine. The commonest shapes include nanorods,[13-19] nanospheres[20-22] and nanoshells,[23-28] but the diversity extends to nanocubes,[29-34] nanowires,[35-38] nanorockets[39] and nanostars[40-44] to name a few. In general, NPs smaller than 100 nm have excellent tumour targeting ability,[45] being small enough to permeate out from porous vascular endothelial fenestrations that surround a region of tumour.

Theranostics refers to agents that are simultaneously therapeutic and diagnostic. Theranostics using NPs implies a robust system which can diagnose, deliver targeted therapy and monitor response.[46] When excited with laser energy with a wavelength that is tuned to the gold nanoparticle's (GNP) specific surface plasmon resonance (SPR), valence electrons on the surface of GNPs exhibit very strong oscillatory energy, which induces high temperatures that are useful for causing localised tissue death. When these NPs are heated within cancer tissue, this is then termed photothermal therapy (PTT). This photothermal reaction can be applied to kill cells within tumours, specifically in places that are difficult to reach surgically or require a palliative debulking procedure. The SPR of GNPs can be tuned to absorb light in the near infrared (NIR) region to harness the potential of applying this photothermal effect to cancer tissue *in vivo*. The first use of GNPs in photothermal ablation was described by Hirsch et al in SKBr3 human breast epithelial carcinoma cells in 2003.[15]

NPs are also being used to deliver therapeutic chemicals directly to tumour sites, by extending their ability to also act as nano-carriers. Formulations of nanoparticles such as Doxil™, Abraxane™, Resovist® and Feridex® are already in clinical practice.[47] Despite this progress, there remain considerable uncertainty and variation in methods and results from the application of GNPs in GI cancer that have been published. It is perhaps this existing uncertainty and variation that has forestalled the transition of GI cancer theranostics from *in vitro* and murine *in vivo* studies to human clinical trials. By applying GNPs that can target GI adenocarcinomas, the thermal effect that would result from irradiation by a light source could exert an ideal therapeutic effect on the cancer tissues. Studies have shown that GNPs have relatively negligible cytotoxicity on healthy cells, making them ideal for cancer-specific therapy.

Thus the aim of this systematic review is to compartmentalise and consolidate the progress of *in vitro* and *in vivo* applications of the most studied inorganic metallic NP- GNP - in GI cancer. This paper aims to highlight and provide some objective evidence into some of the current controversies surrounding their application in the GI tract by discussing published findings relating to their size, shape, synthesis, surface charge, active and passive targeting efficiency, cellular uptake, biocompatibility, drug delivery and most crucially, their toxicity. GNPs have afforded new applications for a host of imaging platforms to enhance optical detection of these cancers, thus these are also reviewed. Where possible this paper attempts to elucidate if there are any potential conclusions that can be drawn on their optimisation, efficacy and safety, and identify any potential issues that need addressing prior to elevating nanomedicine from the bench to clinical practice.

Background

Hyperthermia and photothermal therapy

Upon irradiation of GNPs with NIR light, surface electrons become excited and resonate vigorously. When these electrons return to the ground state, they emit energy in the form of heat and the surrounding temperature is raised.[48] The temperature rise is primarily dependent upon the shape and concentration of the NPs, incubation time of GNP with tissues, laser fluence (power per unit area) and the laser exposure time.[49,50] The characteristic absorption spectrum of GNPs is dependent on the shape of the particles and is usually chosen to be within the NIR spectrum [between 650 and 900 nm for up to 10 cm depth of penetration[51-53]] where there is minimal background tissue absorption and high optical tissue penetration.[46] In the case of gold nanorods (GNRs), altering and increasing their aspect ratio (length/width) during chemical synthesis shifts the absorptive peak of their longitudinal SPR band within the visible and NIR.[54-56] The application of gold nanospheres has rather limited spectral tunability due to their resonance peak at approximately 520 nm in the visible, which thus has a more limited clinical application in GI cancer due to the absorption and scattering of this light by tissue and endogenous chromophores.

GNP heating can also release drugs directly into the site of particle accumulation by de-coupling heat-sensitive chemical bonds to the nanoparticles that act as cargo carriers or vectors. Furthermore, the photothermal effect may be channelled to rapidly transport drugs across membranes and damage DNA and proteins as well as generate oxygen free radicals.[57,58]

Within tissue, hyperthermia encourages higher concentration of drugs to localise within a tumour by increasing regional blood flow. Hyperthermia also works at the cellular level by increasing cellular permeability and enables higher intracellular chemotherapy concentrations.[57] Personalised medicine has given rise to 'activated therapy', namely enzyme-cleavable prodrugs,[59,60] which become active and release the parent drug after interacting with a specific biomarker inside the cell.[61] Nanotechnology has allowed the progression of drug-delivery from bench to clinical application. For example, an albumin-bound 130 nm particle such as paclitaxel (Abraxane®, Abraxis BioScience Inc.) has been approved by the US Food And Drug Administration (US FDA) for metastatic breast cancer.[62] Another FDA-approved nanoparticle-based drug in use is doxorubicin (Doxil), which has been validated in a phase III multiple-myeloma trial and further indicated in metastatic ovarian cancer and AIDS-related Kaposi's sarcoma.[63] Although there have been numerous drug delivery systems throughout the world, very few have made it through the

rigours of the Medicines and Healthcare products Regulatory Agency (MHRA), the European Medicines Agency (EMA) or the US FDA, indicating a formidable "bottle neck" from translating bench to bed-side delivery.[64,65]

PTT using NIR light absorption to elicit thermal damage[46] is an established means of destroying cancer tissue, since tissues heated above a certain thermal threshold undergo various mechanisms of cellular damage[66,67] such as protein structural changes or carbonization of tissues. The term hyperthermia is used when an organ is heated to temperatures between 41 and 45 °C. Hyperthermia can also enhance the efficacy of chemotherapy and radiation-induced tumour damage,[68,69] and there are also positive reports of an enhancement of the photodynamic (PDT) response[70] compared to PDT alone.[71] Hyperthermia is an attractive therapy for it retains a lower side-effect profile than conventional cancer treatments, with the potential of repeated application without the concern of compounding the toxicity levels.[72] One major challenge to local and regional PTT is the development of a homogeneous temperature distribution throughout the tissue,[73] as the heating delivered from lasers generally follows a Gaussian profile. Temperature-dependent cell survival graphs have shown that each 1 °C temperature rise above a 43 °C threshold leads to doubling of cell death.[58]

Techniques which employ temperatures above 45 °C to produce irreversible cell damage are referred to as thermal ablation techniques,[57] such as those used in radiofrequency or microwave ablation. This produces a specific area of cellular death bordered by regions experiencing less intense hyperthermia and potentially viable. Cancer cells appear to be more sensitive to heat-induced damage than normal cells.[74]

Rodent studies demonstrated that tissue depths of approximately 1 cm could be irradiated safely with NIR light using untargeted gold nanoshells with less than 10 °C increases in normal tissues.[25] These results concur with Shah et al demonstrating that NIR wavelengths are able to penetrate to depths of more than 1 cm in tissues without visible damage.[75] Depth of penetration and selectivity of PTT are some of the key challenges encountered in translating this technology to patients, where tumours may be extending 5-10 cm deep within parenchymal structures.[24]

Enhanced permeability and retention effect (EPR) and tumour targeting

First described by Maeda and Matsumura in 1986, the enhanced permeability and retention (EPR) effect provides an explanation for specific accumulation of GNP at the tumour site.[76,77] They explained that NPs selectively accrue within solid tumour masses as a result of tumour physiology. Solid tumours contain leaky blood vessels with cell junction gaps ranging from 100 nm to 780 nm,[78] compared with pore diameters of up to 20 nm in normal capillaries.[79-81] Studies have repeatedly demonstrated that NPs with diameters up to 100 nm will pass through the reticuloendothelial system (RES) and into the circulation to extravasate and accumulate in the tumour region.[79,82-85] However, the sizes of these endothelial fenestrations are known to vary with tumour type and microenvironment.[86] Once assembled inside the tumour interstitium, NPs are retained due to locally ineffective lymphatic drainage. This is a passive method of organising GNPs into cancerous regions, so that they are optimally positioned for PTT. For tumours less than 3 cm, local hyperthermia using targeting derived from passive GNP accumulation may be suitable[58,87] but the biggest limitation is the considerable biological heterogeneity of tumours and hence the lack of bio-specificity. Tumours with poor vasculature, such as pancreatic or prostate cancer, may not amass GNPs via the EPR effect alone.[74]

Active targeting has consequently been explored to enhance the GNP concentration within the tumour matrix by attachment of a targeting moiety that is over-expressed in cancer cells. The GNP surface is modified with an antibody or ligand for receptor, antigen, carbohydrate or other type of targeting.[85,88] Antibodies that have been applied in targeting gastrointestinal cancer include human epidermal growth factor receptor 1 (EGFR), vascular endothelial growth factor (VEGF), folic acid (FA) receptors and vascular cell adhesion molecule-1 (VCAM-1). Two distinct targeting mechanisms may be used to aid tumour specificity.

Following conjugation to a specific receptor, GNPs internalise via the characteristic mechanism for that particular receptor; for example, GNPs targeting EGFR receptors become internalised within 15 minutes of receptor-ligand engagement[68] — see section below. The biggest limitation associated with active targeting is the fact that the GNPs are typically larger and experience difficulty in mass transport across bio-barriers, and also competitive uptake by non-target cell types or extracellularly.[89] This may partly explain why the current GNPs in clinical use utilise passive targeting via the EPR effect rather than active biomolecular recognition, as well as the more complex clinical approval route for targeted agents.[90]

Synthesis and surface coating of GNPs

In 1857 Michael Faraday pioneered the synthesis of colloidal gold; where he described a chemical synthesis of reducing gold chloride in a carbon disulfide solvent using phosphorous as a reducing agent.[91] Today, there are three main methods to synthesise GNPs: physical, chemical and biological. The physical methods of synthesis comprise microwave irradiation,[92] ultra-violet irradiation,[93] laser ablation,[94] sonochemical methods,[95] thermolytic processes,[96] photochemical and radical induced methods.[97,98] The biological method uses fungi or bacteria as nanofactories.[99,100]

In the synthesis of gold nanorods, which are the most widely used GNP in the PTT of GI cancer, the most commonly used method comprises a chemical seed-mediated approach whereby spherical 'seed' NPs (~4 nm) are added to a growth solution containing gold salt, silver nitrate, ascorbic acid and cetyltrimethylammonium bromide (CTAB) leading to the fabrication of GNPs with a rod-like morphology (i.e. GNRs).[101,102] This was first described in the 1920s[103] and is a relatively simple and reproducible method of obtaining a high yield of GNRs with varying aspect ratios.[104]

CTAB, a cationic surfactant coating, induces a positive charge to the surface of GNRs and in an aqueous medium, it prevents particle aggregation due to electrostatic repulsion.[16] CTAB can be cytotoxic as it can cause biomembrane and peptide disintegration at micromolecular concentrations.[105] Therefore, it

is essential to replace or remove the CTAB coating on GNRs in order to effectively apply GNRs in biomedical uses. Attempting to remove excess CTAB from newly synthesised GNRs with successive washings, centrifugation and removing the supernatant CTAB, CTAB-capped GNRs at a concentration of ~200 μg ml^{-1} still exhibited marked cytotoxicity.[106] Thus, it is generally accepted that an outer protective coating on GNRs, such as PEGylation, silica or poly(acrylic) acid (PAA) is essential for most biological applications.[16]

In order to exploit the EPR effect, hydrophobic GNPs must escape systemic recognition by the immune system. Cells of the RES, particularly macrophages, are scavengers that inhibit effective GNP treatment by phagocytosing or opsonising NPs and thus prohibit them from gaining access to tumour cells.[107] Nevertheless, the surface of NPs is easy to modify; and by coating a hydrophilic 'stealth' conjugate such as polyethylene glycol (PEG) onto their surface, the clearance by the RES organs such as the kidney, liver, spleen, and lymph nodes is decreased,[68,85,88] whilst prolonging circulatory half-life by 10-100 fold.[78,108] "PEGylation" of GNPs also provides an external shell for ligand conjugation and prevents particle aggregation. A disadvantage of PEGylation is that it can potentially shield the targeting agent, which reduces the likelihood of biorecognition.[109]

Cellular uptake of GNPs and dependence on GNP type and shape

NPs traversing the GI tract bypass efflux by transmembrane ABC (ATP-binding cassette) transporters and subsequently enter cells via endocytosis.[110] The process of GNR internalisation was studied by Chithrani et al using transferrin-functionalised GNRs. The authors concluded that receptor-mediated endocytosis was the main mechanism behind internalisation based on a 70% decrease in cellular uptake at low temperatures (4 °C), which is known to cease receptor-mediated endocytosis.[111]

It is important to examine the distribution of GNPs in tumours at both tissue and cellular levels. As GNPs are electron-dense, transmission electron microscopy (TEM) or scanning electron microscopy (SEM) are both able to confirm internalisation of GNPs into gastrointestinal cancer cells, observe aggregation as well as characterise the size and shape of the GNPs. EM can also display post-irradiation changes to intracellular architecture and organelles after NIR light absorption by intracellular GNPs. It can also be utilised to quantify non-selective uptake of GNPs by non-cancerous cells and the collateral spread of PTT damage to adjacent healthy tissues. Inductively coupled plasma-mass spectrometry can be used to give a precise quantification of the amount of administered gold which has been taken up by cells or tissues.[105,112]

There is some debate as to whether gold nanospheres, gold nanoshells or GNRs (the three most widely applied GNPs in the PTT of GI carcinoma) are preferable for biomedical targeting and delivery, as they all employ the same principle of SPR to release thermal energy to the surrounding tissues. Gold nanoshells (approximately 10-300 nm in diameter) comprise a dielectric core, usually silica, which is encompassed by a thin gold shell.[25,26] Huang et al found that when targeted gold nanospheres and GNRs were compared with each other in terms of receptor binding to malignant oral epithelial cancer cells, many more GNRs appear to bind to malignant cells due to interactions between the surface of the rods and cell surface proteins.[15] Huang also pointed out that on some occasions GNRs also accumulated in non-malignant cells due to non-specific interactions. von Maltzahn et al further demonstrated that PEG-GNRs were superior to PEG-gold nanoshells in terms of intrinsic absorption and photothermal efficacy (GNRs generated more than 6 fold greater heat per gram of gold), as well as significantly longer circulation times *in vivo* (~17 hours for PEG-GNRs versus ~4 hours for PEG-gold nanospheres), which may be attributable to their polymer coating.[113]

Chen et al evaluated GNP size-associated toxicity over time. They found that GNPs ranging from 8 to 37 nm produced severe sickness in mice and side effects including fatigue, anorexia, fur colour changes and weight loss. The majority of mice injected with these sized GNPs died before the end of the fourth week.[114] It is important to mention that the GNPs used were not PEGylated, rather somewhat unconventional surface modification peptides (pFMDV and pH5N1) were utilised. The authors also observed that very small GNPs (5 nm) or larger (50-100 nm) were in fact non-toxic.

Desai et al explored the relationship between GNP size and GI tract uptake and showed that GI cell endocytosis occurs more readily when NP sizes are below 130 nm.[115] It is thus presumed that both active and passive targeting can be capitalised simultaneously to maximise the efficacy of GNP targeting, with the proviso that the combined particle-conjugate size remains approximately 130 nm or smaller to avoid uptake by the RES.

NPs have a large surface area to volume ratio which allows them to be held in suspension, incorporate targeting moieties, allow high pro-drug encapsulation and high loading capacity for imaging probes, but also permit extensive surface absorption.[61,116-118] It is known that most chemotherapy drugs distribute non-specifically within the body, which accounts for much of its toxicity and side effects. However GNPs loaded with cleavable pro-drugs are able to specifically internalise within cancer cells. This presents an elegant solution to the problem of non-specific biodistribution and poor bioavailability of conventional drugs.

Positively charged nanoparticles (from zeta potential measurements) were believed to be more likely to adhere to negatively charged cell membranes[119-121] by electrostatic interaction. However, doubt remains as to what extent the charge of GNPs influences the rate of cellular uptake.[122] Arvizo et al suggested that cell membrane potential significantly affects the uptake of GNP, and showed that cationic GNPs were much more efficient at depolarizing the membrane and thus being taken up by both cancer and healthy cells, compared with anionic or neutral GNPs.[123] Lund et al were more sceptical of this theory and proposed that it is more likely NPs either enter through pre-existing cell membrane pores or are capable of re-configuring the plasma membrane in order to create new pores.[122] They used very small NPs (5 nm) and proposed passive internalisation by pathways which do not depend on energy, endocytosis or lipid-raft-mediated methods. Alkilany et al studied the cellular uptake of differently charged GNRs, and found that particle surface charge bore no correlation to GNR uptake.[105] Zahr et al proposed that the higher the surface charge of a GNP, regardless its polarity, the more likely it is to be phagocytosed by macrophages and removed from the circulation.[124] In practice nanoparticle surface charge is often

minimised by the incorporation of a neutral polymer such as PEG which limits electrostatic interactions with other components within the circulation.

Imaging modalities and diagnostics

Imaging the location of GNPs enables the potential diagnosis of cancer as NPs can be targeted to cancer using both the active and passive approaches discussed above. It is also important to image the location of the NPs to understand their biodistribution and to target the laser to this precise location to gain a high level of specificity for directed therapy.

As gold nanoparticles are an excellent optical contrast agent (primarily through optical absorption in their SPR wavelength bands as well as their intrinsic luminescence under two-photon excitation) they may be imaged using imaging techniques which utilise this property, i.e. two-photon luminescence imaging,[125-127] photoacoustic imaging,[126,128] narrow band imaging[129] and optical coherence tomography (OCT).[13,130]

NIR fluorophores such as Cy5.5 may be conjugated to the surface of NPs for background-free diagnostic fluorescence imaging to clearly localise aggregates of GNPs within tissue. Once fluorescence is identified within a cluster of nanoparticles, NIR laser illumination may then be directed to that location for PTT.

Non-optical methods have also been used with GNPs such as positron emission tomography (PET) and x-ray computed tomography (CT). They have been used with X-rays as gold has a higher atomic number and density compared to standard radiosensitive iodine-based reagents.[131,132] von Maltzahn et al have shown preliminary evidence that GNRs appear to exhibit approximately two times more X-ray contrast than that of standard iodine per mole.[113] Gold nanoshells[133,134] and nanocages[135] have also been attached with the radionuclide ^{64}Cu to enable PET imaging of the NP location.

Materials and methods

This systematic review was performed in accordance with guidelines from the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA).[136]

Eligibility criteria

Original peer-reviewed articles published in English on the application of GNPs in GI tract cancer (including oesophageal, gastric and colorectal carcinoma, but excluding oral, hepatic or pancreatic cancers) were considered. Studies using NPs without any gold element were excluded. Where multiple studies existed from the same institution, the most recent study was considered.

Information sources and search

A broad literature search was conducted in May 2013 using PubMed (1946 to date), Embase (1974 to date) and PsycINFO (1967 to date) databases. Additional searches using the Cochrane Library, Ovid SP and cross-referencing with Web of Science® were used to broaden the search. The MeSH search terms used were "gold nano*" and "*esophag*" or "gastr*" or "colo*" or "rectal" or "*intestinal" and "cancer".

Study selection and data collection process

Two reviewers (M.S. and D.S.E.) independently reviewed all relevant articles from the literature search. The full text of each article was obtained and further screened for inclusion if it had relevance to application of GNPs in GI tract cancer. Studies were excluded if they were only conference abstracts without any extension to a full supporting paper due to the lack of data and methods, and studies were excluded if they only were on hepatic or pancreatic cancer. A high level of agreement existed between both reviewers, and minor queries were discussed between the reviewers until a 100% concordance was achieved on the final studies included in this review.

Data items

The following items were extracted from the studies: GNP type, shape, average size and concentration used, type of cancer cell lines or animal tumour model used (or both), charge of GNPs, employment of targeting agents, methods of confirming intracellular accumulation of GNPs, laser radiation type, fluence and regime, confirmation of PTT effects and temperature rises, confirmation of histological evidence of cellular destruction or cell viability studies (for cell studies), survival studies or follow up (for animal studies), imaging modalities used, and any evaluation of toxicity.

Results

Initial searches using the MeSH terms above revealed 284 articles. There were nine conference abstracts (without accompanying full papers), which were excluded. A further 48 articles were identified through free text searches, the "related articles" feature and cross-referencing. Once duplicates were removed, finally 16 studies remained and were found to match the inclusion criteria, thus these are discussed in this systematic review.

GNP type and concentration

GNPs that were used in the theranostics of GI tract cancer involved a combination of GNPs conjugated with silica,[137,138] PEG,[23,129,139,140] chitosan,[141,142] iron core (with a gold shell [143], pure shells,[144] platinum-tethered,[145] CTAB-coated GNR,[146] gold-SPION hybrid NPs,[147] PEG-conjugated hyaluronic acid NPs,[148] PEG-Au-TNF[149] and poly(acrylic acid)-GNR.[106] Although there are many shapes of NPs in existence, the three identifiable GNP shapes were rods, shells and spheres. The other identifiable characteristics of the studies are described in Table 1.

Charge of NPs

Zhang et al measured 15 nm chitosan-coated GNPs using zeta potentials, and found they bore a charge of $+30.0 \pm 1.18$ mV at a pH of 7.4.[142] They proposed that the positive charge promotes particle repulsion and prevents agglomeration, whilst enhancing endocytosis when interacting with negatively charged cell membranes. Huang et al also measured the zeta potential of their synthesised GNPs, but it was unclear whether the authors considered this to have a bearing on GNP internalisation.[137]

Table 1
The included studies, with the type of study, shape, size and concentration of GNPs used in the study.

Study	Cells/animals	Shape	Ave. size (nm)	GNP concentration
Huang et al[137]	Cells + mice	Rods	46 × 18	0.625-12.5 μM
Sazgarnia et al[144]	Mice	Spheres	6-8	38.6 μg/ml
Li et al[141]	Cells + rats	Particles	30-90	OD 1 (cells) or 3×10^{11} NP/ml OD 50 (animals)
Wu et al[143]	Cells	Particles	10	10 μg/ml
Zhang et al[142]	Cells	Particles	15	20-100 μM, OD 0.6
Puvanakrishnan et al[129]	Mice	Shells	135	2.66×10^9 NP/ml, OD 1
Goodrich et al[140]	Mice	Rods	45 × 14	4.5 ml/kg or OD 100 given IV to mice (2×10^{13} GNR/ml)
Brown et al[145]	Cells	Particles	30-40	?
Black et al[146]	Cells	Rods	60 × 20	?
Kirui et al[147]	Mice	Hybrid particles	6-18	PTT — 200 μL, 1 mg/ml MRI — 0.3 ml, 1 mg/ml
Gobin et al[23]	Mice	Shells	119	150 μL (1.5×10^{11}/ml)
Choi et al[148]	Mice	Particles	238	?
Paciotti et al[149]	Mice	Particles	33	5-24 μg
Diagaradjane et al[139]	Mice	Shells	132-135	8×10^8/g body wt.
Kirui et al[106]	Cells	Rods	66 × 11	100 μg/ml
O'Neal et al[138]	Mice	Shells	8-10	100 ml of 2.4×10^{11} NP/ml solution

Key: NP = nanoparticle, OD = optical density, ? = unknown/unclear.

Passive or active targeting

Twelve (75%) of the gastrointestinal cancer studies did not involve functionalisation with a targeting agent, relying instead solely on the EPR effect of passive accumulation of GNPs intracellularly and into the tumour tissue.

Folic acid was used as a targeting agent for MCG803 gastric cancer cells.[137] Kirui et al adopted immuno-targeting using humanised single-chain antibody conjugates (A33scFv) that target the A33 antigen expressed in 95% of primary and metastatic human colorectal cancer (CRC) cells, but is absent in most other normal tissues and tumour types.[106,147] Hyaluronic acid receptor (CD44) that is over-expressed in various cancer cells[148] has also been employed for targeting.

Cancer models used

The cancer models used were broadly categorised to either cellular studies (*in vitro*) and/or animal studies (*in vivo*) as shown in Table 2.

Irradiation regimes

The irradiation regimes used with gold nanoparticles are shown in Table 3.

Proving endocytosis of gold nanoparticles

A variety of different methods were used to identify the uptake of GNPs into cells and tissues. Almost all studies employed TEM imaging to visualise nanoparticles post synthesis, but three studies also used it to visualise NPs within cells[137,142,145] and one study used dark field microscopy.[137] It was found that GNRs are virtually unchanged after internalisation and it is apparent that GNPs do not enter the nucleus, but agglomerate within intracellular vesicles.[137,142] The uptake and localisation of platinum-tethered NPs were also examined using inductively coupled plasma mass spectrometry, which confirmed the ability of GNPs to deliver platinum inside cells.[145]

Fluorescent protein labelling of a colon cancer cell line was used by Black et al[146] whilst Kirui et al used NIR fluorescence imaging of localised intratumoural gold-SPION hybrid NPs.[147] In a prior study, Kirui et al[106] also showed that human CRC SW 1222 cells incubated with fluorescently-labelled A33scFv-GNRs had internalised into cells using fluorescent-based confocal microscopy analysis. Gobin et al excised tumours and then cryosectioned them with silver staining prior to microscopic analysis,[23] confirming that nanoshells were present throughout the tumours. Li et al similarly demonstrated GNP loading in cells via histology using silver staining.[141]

A method used to identify iron-gold hybrid GNPs within cancerous tissues was using Perls' Prussian blue staining.[147] Other excised tumour sections were lyophilised for gold content evaluation using neutron activation analysis,[23] which was able to verify the presence of nanoshells within the tumour.

Photothermal effect, hyperthermia and cancer cell destruction

Photothermal effects were evaluated in all studies that involved laser application. However, in one study suppression of cancer cell proliferation was noted without laser illumination, which was attributed to the GNP composition causing local cytotoxic effects. Wu et al noted that iron clusters before oxidation in their iron core-gold shell nanoparticles specifically inhibit the growth of human CRC cells (CaCo-2 & HT-29), leaving healthy cells unaffected.[143]

Ultrasound (US) irradiation alone showed an insignificant anti-tumour effect as shown by Sazgarnia et al. However, they showed that acoustic cavitation in the presence of GNP with intense pulsed light (IPL), a broadband (560-1200 nm), pulsed, high energy light source, could be used as a new method to improve therapeutic effects on tumours.[144] The authors discovered that tumour inhibitory effect was significant when IPL and US and GNPs were used. They hypothesised that IPL

Table 2
Types of *in vitro* and *in vivo* GI cancer models and methods of inducing cancer in rodents.

Study	Cells	Animals	Cell line on animals	Inoculation method
Huang et al[137]	MCG803 human gastric cancer	Nude mice	MCG803 gastric cancer	Flank s/c
Sazgarnia et al[144]	No	BALB/c mice	CT26 colon carcinoma tumour	Flanks s/c
Li et al[141]	Het-1A, BAR-T and OE-19 human oesophageal lines	Sprague–Dawley rats		Esophago-duodenal anastomosis
Wu et al[143]	Caco-2 and HT-29 human colon cancer cells	No		
Zhang et al[142]	Gastric cancer MGC-803 and human gastric mucosa epithelial GES-1 cells	No		
Puvanakrishnan et al[129]	No	Swiss nu/nu mice	HCT116, ATCC#CCL-247 human colon cancer cells	Flank s/c
Goodrich et al[140]	No	Balb/c mice	CT26.wt murine colon carcinoma (ATCC)	Flank s/c
Brown et al[145]	HCT116, HCT15, HT29, RKO human colon cancer cells	No		
Black et al[146]	HCT-116 human colon cancer cells	No		
Kirui et al[147]	No	Balb/c nude mice	a) SW1222 cells (antigen-expressing human colorectal cancer cell line).	Left flank s/c
			b) Human colorectal cancer cell line (HT-29)	Right flank s/c
Gobin et al[23]	No	BALB/c mice	CT-26, ATCC murine colon carcinoma cells	s/c
Choi et al[148]	No		3 models:	
		BALB/c mice	HT29 human colon cancer cells	1×10^7 HT 29 cells in 100 ml saline s/c into mice dorsa.
		BALB/c mice	Liver-implanted with CT26 colon cancer cells	Laparotomy & direct injection of 3×10^5 CT26 cells into the left liver lobe.
		A/J mice	Azoxymethane (AOM)-induced orthotopic colon cancer models.	Intraperitoneal injection
Paciotti et al[149]	No	C57/BL6 mice	MC-38 colon carcinoma cells	s/c
Diagaradjane et al[139]	No	Nude Swiss mice	HCT 116 human colorectal cancer cells	$\sim 2 \times 10^6$ cells s/c into right thigh.
Kirui et al[106]	SW 1222 (10^6 cells/ml) human colorectal cancer cells	No		
O'Neal et al[138]	No	BALB/c AnNHsd Sprague–Dawley mice	CT26.WT murine colon carcinoma tumour cells (ATCC)	s/c into flank

Key: s/c = subcutaneous injection.

irradiation on GNP enhances antitumour effects by establishing nucleation sites for acoustic cavitation.

Huang et al noted that gastric cancer cells incubated with GNR-SiO_2-FA, destroyed cell spindle morphology, ruptured cell membranes and produced significant scarring after 3 minutes of NIR laser (4 W/cm^2) application.[137] X-ray irradiation was also utilised on chitosan-modified GNPs (CS-GNPs), and the survival fractions of gastric cancer cells treated with CS-GNPs decreased when increasing the concentration of CS-GNPs and when compared to cells without CS-GNPs under the same X-ray radiation dose.[142] Kirui et al proved effective PTT of CRC cells that had been incubated with plasmon-resonant A33scFv-GNRs and treated with NIR laser (5.1 W/cm^2) for 5 minutes.[106]

In measuring local tissue temperatures achieved from PTT, Goodrich et al noted that in a mouse study, the average maximum temperature difference for GNR-infused and laser-treated animals was approximately 32.1 ± 9.0 °C. In tissues undergoing GNR-assisted laser PTT, they observed maximum temperatures of approximately 62.0 ± 9.0 °C in tissues, whilst with the laser-only control animals the maximum tissue temperatures were approximately 45.3 ± 2.8 °C. These temperature rises were noted over a 3-minute NIR laser (3 W) irradiation period.[140] Kirui et al noted a 30 °C temperature rise for a concentration of 0.5 mg/ml hybrid NP using a regime of 7 rounds of NIR CW irradiation (5 W/cm^2) over a fortnight.[147] O'Neal et al demonstrated after 30 seconds of NIR irradiation (4 W), the average temperature of laser-nanoshell treated colon cancer in mice was approximately 50 °C and this was statistically significantly higher than the nanoshell-free (but NIR irradiated) controls. A complete tumour resorption was seen after 10 days of laser-nanoshell treatment.[138]

Diagaradjane et al used H & E (haematoxylin & eosin) to demonstrate there were necrotic regions at a distance of ~1.4 mm from the tumour periphery in their thermoradiotherapy group, which showed a distortion of regional architecture characterised by patchy hypoxic regions in the tumour core with no identifiable regions of blood flow.[139]

Histological evidence of destruction and cell viability studies

The effects of PTT on cells using GNPs should be evaluated to ensure selective cellular destruction of cancer cells and the

Table 3
Irradiation regimes used in each study model.

Study	Cells/animals	Irradiation used	Laser power & fluence	Duration of radiation
Huang et al[137]	Cells & mice	CW laser 808 nm	30 mW laser power 4 W/cm^2 laser fluence	3 mins
Sazgarnia et al[144]	Mice	Intense pulsed light (IPL) (LumenisOne), a broadband (560-1200 nm), pulsed, high energy light source + US	US: 2 W/cm^2, with frequency 1.1 MHz Light: 35 J/cm^2	US — 3 mins IPL — 9 pulses of 5 ms pulse duration
Li et al[141]	Cells & mice	CW laser 818 nm — used both externally & via microendoscopy	3 W/cm^2	Cells & rats, 1 min at 3 W/cm^2, or, 30 sec, 1 W/cm^2
Zhang et al[142]	Cells	X-rays	1 Gy/min	Cells exposed to 2, 6 & 10 Gy with corresponding irradiation times of 2, 6 & 10 min
Goodrich et al[140]	Mice	CW laser 808 nm	3.5 W at 4.46 W/cm^2	180 seconds
Black et al[146]	Cells	Ti:Sapphire at 800 nm	1 mW (imaging), >10 mW for PTT with beam diam. approx. 20 μm	No time duration specified just states “4 passes”
Kirui et al[147]	Mice	CW laser 808 nm	5 W/cm^2, 6 mm diam	30 mins & 7 rounds therapy over 14 days
Gobin et al[23]	Mice	CW laser at 808 nm	4 W/cm^2, spot size 5 mm	3 mins
Diagaradjane et al[139]	Mice	808 nm CW laser + a single 10 Gy dose of radiation therapy using 125 kV X-ray operated at 20 mA	0.6 W used, 75% duty cycle, average optical irradiance (350 mW/cm^2) 10 mm diam	20 minutes
Kirui et al[106]	Cells	CW laser 808 nm	5.1 W/cm^2 with beam size 4 mm diam	10 mins
O’Neal et al[138]	Mice	CW laser 808 nm	4 W/cm^2, 5 mm diam	3 mins

Key: CW = continuous wave, US = ultrasound, Gy = Gray (joule/kg).

viability of healthy surrounding tissues post irradiation. Studies used a variety of methods in this endeavour to demonstrate cytotoxicity or apoptosis, chiefly using trypan blue staining,[137,146] H & E staining and microscopy,[139-141,144] Annexin V-fluoroisothiocyanate (FITC) apoptosis detection kit I,[143] ApopTag® apoptosis detection kit[141] and assays such as WST-1,[143] CCK-8,[137] MTT,[141,142,145] TUNEL[141] and clonogenic cell survival assays.[142] The clonogenic cell survival assay is an *in vitro* assay based on the ability of a single cell to reproduce to form a colony after ionising radiation, i.e. its survivability.[142] The MTT assay is a quantitative colorimetric method to evaluate cytotoxicity whilst trypan blue is an *in vitro* cytotoxicity assay that measures cell membrane integrity.

Brown et al evaluated the cytotoxicity of platinum-tethered GNPs (as a chemotherapy nanovector) against traditional PEGylated GNPs on human colon cancer cell lines using a tetrazolium dye-based microtitration assay, an MTT assay and inductively coupled plasma-mass spectrometry. Tetrazolium salt assays measure mitochondrial activity. Whilst the PEGylated NPs showed no cytotoxicity, the platinum-tethered gold nanoparticles in contrast were found to be 5.6-fold more cytotoxic than oxaliplatin.[145] Another study also endeavoured to determine cellular viability using 0.5% trypan blue, a dye that does not penetrate the cytoplasm of viable cells, which were added prior to laser treatment of CTAB-coated GNR on a human colon cancer cell line, HCT-116.[146] After 10 minutes of illumination, it was noted that trypan blue had entered several cells within the laser region, and after 25 minutes, the entire irradiated region which had initially absorbed GNRs was stained with trypan blue, whilst other control regions remained unchanged. A subsequent wash of the stained cells on a slide led to the complete removal of thermally affected cells, suggesting major cellular damage.

PEG-conjugated hyaluronic acid nanoparticles (P-HA-NPs) that contained the anticancer drug irinotecan (IRT) were studied on 3 BALB/c mice colon cancer xenografts. It was noted that IRT released gradually from NPs within 12 hours and then exerted a dose-dependent cytotoxicity on colon cancer cells.[148] Kirui et al conducted cell viability studies using an MTT assay of SW 1222 cells (an antigen-expressing human CRC cell line) after incubation with increasing concentrations of polyacrylic acid-GNRs against CTAB-GNRs. A dose-dependent toxicity was noted with a significantly higher cytotoxicity for cells which were incubated with CTAB-GNRs.[106]

Survival studies and tumour regression — in vivo animal studies

This review considered all longitudinal survival studies and tumour volume regression. Sazgarnia et al[144] continued follow-up for 70 days after IPL + US + GNP treatment and noted the survival fraction of these mice was the most significant compared with other control groups. In a different study involving mice inoculated with CT26.wt murine colon carcinoma, the mean survival time with various treatment modalities was established. For the ”no treatment” group, mice lived for an average of 8 days, whilst mice in the “laser illumination only” group lingered for an average of 9.5 days, whilst the “NRs-only” group survived for 9.7 days. Most significantly, it was the photothermal ablation group of mice that lasted longest at 42.1 days.[140] 44% of the GNR and laser-treated mice survived at day 60, together with evidence of complete tumour ablation. It was observed that the mean survival time of the photothermally-treated group was statistically higher than the control groups. O’Neal et al observed colon tumour size and survival for 90 days following a single NIR irradiation treatment in mice receiving IV gold nanoshells. At 90 days post-treatment, 100% of the gold

nanoshell irradiated mice remained healthy and free of tumours. However, tumours in both sham and control groups continued to develop rapidly.[138]

In a study by Gobin et al, tumour size and animal survival were monitored 7 weeks after NIR treatment in CRC induced in mice that were subjected to PEGylated gold nanoshells. All but two nanoshell-treated mice had complete tumour regression.[23] A 14 day median survival was observed in the "saline + laser group", and 10 days for the "no treatment" control group. After 21 days, the group with the most statistically significant survival was the "nanoshell + laser" group, which continued until the end points of the study. It was noted that the median survival time could not be calculated for this group, as the long-term survival was 83%. In a drug-delivery study, Choi et al[148] used the anticancer drug irinotecan (IRT) attached to PEG-hyaluronic acid nanoparticles (P-HA-NPs) on 3 mice bearing CRC xenografts. Tumour volume and survival rates were determined after IRT-P-HA-NPs were given intravenously every 3 days. The authors found that the "saline only" and "free IRT" groups experienced a rapid and significant increase in tumour size and growth. In contrast, significant tumour growth suppression was observed in the group treated with IRT-P-HA-NPs. 50% of mice treated with "free IRT" died after 15 days, and approximately 90% of mice in this group perished within 28 days, indicating IRT by itself results in severe systemic toxicity. Nonetheless the group treated with IRT-P-HA-NPs (using GNPs as a nanovector to deliver the drug into cells) exhibited a much higher survival rate than all control groups.

Imaging modalities

Huang et al evaluated the Hounsfield units (HU) of GNR-SiO_2 by CT. Nude mice implanted with gastric cancer MGC803 cells were selected as the animal model and X-ray imaging was used to monitor the targeting ability of GNR-SiO_2-FA into tissues.[137] Puvanakrishnan et al used NIR narrow band imaging in Swiss nu/nu mice inoculated subcutaneously with human CRC cells to image the accumulation of PEGylated gold nanoshells at the tumour site.[129] NIR narrow band imaging was performed *ex vivo* on excised tumour tissue, and in 4 of 5 gold nanoshell-injected mice, the gold nanoshell regions were visible as dark areas.[129] Kirui et al implanted two colon cancer cell lines subcutaneously in murine models and injected intravenous targeted gold-SPION hybrid nanoparticles (HNPs)-A33scFv and scanned the mice in a 7-T scanner. As a MRI agent, HNPs which had accumulated in subcutaneous CRC reduced the post-contrast T2 phase value by half.[147] Gobin et al used OCT imaging to evaluate PEGylated gold nanoshells in murine CRC. The results showed no enhancement in layers of normal tissue in mice treated with nanoshells, but there appeared to be a significantly enhanced brightness in the region where nanoshells accumulated within a tumour, suggesting that gold nanoshells are able to provide substantial contrast in OCT imaging.[23]

Toxicity of gold nanoparticles

Huang et al showed using a CCK-8 assay that there was negligible cell death and physiological changes in MGC803 gastric cancer cells after exposure to GNR-SiO_2-FA. Even with the highest concentration of GNR-SiO2-FA, cell viability was greater than 90%, indicating that their GNPs were by themselves non-cytotoxic to MGC803 cancer cells within the concentration range studied.[137] Similarly Zhang et al evaluated the cytotoxicity of chitosan-modified GNPs (CS-GNPs) to MGC803 (gastric cancer) and GES-1 (human gastric epithelium) cells using the MTT assay. The cell viability of MGC-803 cells and GES-1 cells was more than 90% even when the concentration of CS-GNPs was increased to 100 μM, and no decline from this high survival rate was seen even after increasing the incubation time to 72 hours, implying very low levels of cytotoxicity.[142]

Li et al showed that their chitosan GNP (CS-GGS) only heated and caused PTT in the presence of NIR irradiation when absorbed by cancerous oesophageal cell lines (OE-19). They induced orthotopic oesophageal cancer in rats four months after forming an oesophagoduodenal anastomosis.[141] The same GNP-laser combination did not have any effect on benign human squamous oesophageal epithelium cells (Het-1A) or Barrett's epithelium (BAR-T). However, the authors cautioned about selectivity of therapy, as they found some regions in the oesophageal mucosa that included both cancerous and adjacent healthy tissues which were "burned" on exposure to NIR. They postulated that this could be due to infiltration of adjacent tissues by inflammatory cells such as phagocytes, and advised that further evaluation of the specificity of GNP uptake in cancerous and benign tissues is required.

Goodrich et al conducted biodistribution studies in twelve mice receiving infusions of high concentrations PEG-GNRs (optical density of 50 or 6.5×10^{12} GNR/ml, giving 6 ml/kg body weight). At one, seven and 28 days post-infusion, some mice were sacrificed and blood and major organs (namely brain, heart, lungs, kidneys, liver, spleen and lymph nodes) and representative tissue samples were harvested for neutron activation analysis to determine gold content. They found concordance with other published results about the clearance and accumulation of GNPs by the organs of the reticuloendothelial system. The largest accumulation was found in the liver and spleen, where 75% of the total injected nanoparticles were noted 24 hours post injection, with negligible accumulation of gold in other organs. There was a gradual clearance of the GNRs from the liver over the 28-day study. Reassuringly there were no signs of acute toxicity from GNRs even at 60 days.[140]

Choi et al used PEG-conjugated hyaluronic acid nanoparticles (P-HA-NPs) loaded with the anticancer drug irinotecan (IRT) on three mice with colon cancer. Microscopic examination of major organs and tumours using H & E staining suggested that IRT-P-HA-NPs was effective at destroying tumour tissues, but only piecemeal necrosis was observed in the liver tissues.[148]

Paciotti et al[149] evaluated tumour volume regression resulting from various TNF treatments and treatment efficacy by varying the doses of TNF given either in its native form or colloidal-gold bound TNF (cAu-TNF) preparations. They noticed that at a dose of 24 μg of pure TNF per mouse, all the mice died, yet at the same dose of colloidal-gold-TNF preparation, not only was there a significant tumour volume reduction, none of the mice became sick or perished.

Summary of evidence

Gold nanoparticle type and concentration

With the exception of one study that used GNPs of 238 nm,[148] all other studies have used GNPs (of different shapes) below

135 nm in diameter/length. These sizes concur with other published studies looking at optimising the EPR effect, including Desai et al who looked specifically at GI tract uptake.[115] There have been a variety of differently shaped particles being utilised in gastrointestinal cancer targeting, including spheres, rods, shells and seven studies which only mention "nanoparticles", which are uncategorised as the final shape is not described.

It is difficult to categorise the concentrations of GNP solutions used, as most studies have not used a standardised system to report the concentrations used. There is a wide disparity in the optical densities (OD) of GNPs used in mice studies, some using concentrated solutions with an OD of 100 whilst another used an OD of 50[141] or an OD of 1.[129] Two studies[23,138] used virtually the same concentration of nanoshells in their mice studies, however they used vastly different volumes. They also had different sized nanoshells, which present too many confounding factors to make the concentrations of various GNPs a comparable entity between studies. It therefore becomes impossible to elucidate an effective or optimal dose for cancer therapy, or to even establish a safe recommended dose. None of the studies ventured to quantify what dose may potentially be lethal or harmful *in vivo*.

Charge of nanoparticles

There has really been only one proponent of maintaining a positive GNP charge,[142] which, in itself suggests that charge is unlikely to be of any consequence. It appears that most GI cancer uptake studies rely primarily on the passive efflux from endothelial fenestrations via the EPR effect, and secondarily using biochemical targeting agents.

EPR effect — passive targeting

Most studies 12/16 (75%) did not involve GNP functionalisation with a targeting agent, solely utilising the EPR effect for tumour localisation. Goodrich et al state that their previous experience using the concept of EPR for assessing the biodistribution of infused GNP to tumour found that less than 10% of the total injected dose actually reaches the tumour.[140]

Biological agents — active targeting

Folic acid was used for targeting gastric cancer cells[137] whilst the A33 antigen[106,147] and hyaluronic acid receptor[148] were used in targeting CRC. It remains to be proven if there is a definite combination of active and passive targeting that would provide ideal cancer targeting, but this would need to be balanced against the risks of provoking heat-induced bleeding or perforation if applied on more advanced (T3 or T4) cancers.

Cancer models used

There have been eleven *in vivo* rodent experiments, mostly with superficial tumours inoculated through subcutaneous injection of cancer cell lines in rodents.[23,129,137-141,144,147-149] Other ways of inducing cancer include intraperitoneal injection of azoxymethane (AOM) in A/J mice. AOM treatment is used to induce colonic tumours as it mimics the adenoma-to-carcinoma sequence of CRCs in humans.[148] Oesophageal cancer was induced orthotopically by mucosa-to-mucosa anastomosis between the lower oesophagus with the duodenum.[141] In this study, GNPs were sprayed onto the surface of suspicious oesophageal mucosa via microendoscopy. The authors chose to do this as they cautioned that there is a real risk of GNPs becoming trapped in the interstitium of benign tissues with direct injection of GNPs.

The most common gastric cancer cell line used to induce cancer in murine models was MCG 803, whilst a variety of different cell lines were used to induce CRC, including (in order of popularity) CT26.wt (ATCC), HCT 116, HT-29, MC-38, SW1222 CRC cell lines. With regards to cancer cell studies, there is only one oesophageal study,[141] one gastric cancer[142] and four colon cancer ones.[106,143,145,146] Overall, there has been a lack of published studies using the same cancer model, laser regimes, fluorophores and GNPs (including similar concentrations) to make a valid and objective appraisal about the ideal protocol for a particular tumour type or the extent of its reproducibility. This is perhaps one key element that has hindered the progression of GNPs in clinical trials for GI cancer, whereas trials in other cancers have gone the distance. It highlights a need for more vigorous reporting on these key elements.

Irradiation regimes

Vital pre-requisites for successful PTT include particle accumulation and appropriate laser dosimetry at an appropriate balance, for too high a laser exposure will entail excessive heating and collateral tissue damage, whilst too low a laser dose may mean incomplete ablation.[140]

For cell studies, a mean laser fluence of 4.0 ± 1.1 W/cm^2 was used, whilst in rodents, a mean of 3.5 ± 1.7 W/cm^2 was applied for PTT. Most studies involving PTT used CW NIR laser, except one relying on acoustic cavitation induced by US (which also used intense pulsed light), two studies used X-rays and one a Ti:Sapphire laser. It is vital to accurately measure the thermal energy being delivered and the heating occurring, either with a thermal imaging camera or a thermocouple. Equally crucial is the laser beam diameter and distance from the tip of the laser fibre to the tumour's surface for a real appreciation of the fluences required for the thermo-ablative responses seen.

Photothermal effect, hyperthermia and cellular destruction

Whilst CW NIR lasers have been applied in eight studies for PTT and hyperthermia in GI cancer cells and tissues, only three have mentioned the temperature peaks achieved. One study used intense pulsed light in combination with US irradiation and GNP as a novel way to gain the therapeutic effect. X-ray irradiation was also used effectively in conjunction with CS-GNPs for thermal destruction of gastric cancer cells. The maximal temperatures obtained by irradiating *in vivo* GI cancer tissues in the presence of GNPs ranged from 50 to 62 °C, but the three studies comprise different GNPs, concentrations and laser power. From these GNP studies, the mean laser fluence required to heat tissues to this temperature range was 4.5 W/cm^2.

It remains debatable whether there is a time-dependent peak of intracellular GNP concentration giving rise to an optimal therapeutic window for laser application. This issue should be addressed in future studies, and would involve imaging GNPs at various time points.

Proving endocytosis of gold nanoparticles

There is consensus that once internalised, GNPs do not enter the nucleus, but aggregate in vesicles within the cell. TEM is the most commonly used imaging modality employed to determine the size, shape and intracellular location of GNPs. Other techniques shown to be applicable for imaging GNPs within GI cells and tissues include dark field microscopy, inductively coupled plasma mass spectrometry, fluorescence protein labelling and imaging, fluorescent-based confocal microscopy, silver staining, Perls' Prussian blue staining (for iron-gold hybrid NPs) and neutron activation analysis.

Histological evidence of destruction and cell viability studies

All studies used a variety of methods to assess cell viability after cell or tissue treatment with thermoradiation. The three most commonly used methods to demonstrate cytotoxicity or apoptosis were H & E staining and microscopy, followed by MTT assays and trypan blue staining.

Survival studies/follow-up — in vivo animal studies

Five studies presented longitudinal data from the application of GNPs and PTT. Two of these suggested that all murine models of CRC survived and nearly all had complete tumour regression after GNP and irradiation treatment.[23,138,148] Where survival was studied, it is without doubt that the group of animals which received the combination of GNPs and laser lived the longest, and their survival was always statistically significant compared to other interventional arms.[140,144,148] It is thus encouraging that when applied *in vivo* as a therapeutic modality for GI cancer, the GNP and NIR combination appears effective at regressing tumour and prolonging survival. This is the single most important therapeutic information that is consistently demonstrated in this review, and could potentially establish a firm foundation for clinical translation.

Imaging modalities

GNPs have potential as X-ray and CT contrast agents due to their ability to induce strong X-ray attenuation[150] and are actively being investigated as a radiosensitiser. Within GI cancer, GNPs have been used as contrast agents in imaging modalities as diverse as MR, OCT, NIR narrow band imaging and CT. The images obtained can also be used to monitor targeting and response to treatment. Kirui et al synthesised iron-gold hybrid nanoparticles (HNP) and suggested that the iron oxide portion of the HNP served as the MR imaging agent, whilst the gold NP portion formed the hyperthermia agent.[147]

Toxicity of gold nanoparticles

Data obtained from a host of methods including CCK-8, MTT assay, inductively coupled plasma mass spectrometry, neutron activation analysis and microscopy show no apparent cytotoxicity of GNP on cancer or healthy cells without the use of laser irradiation. This is important to know as there may be non-specific binding of GNP to non-cancerous cells/tissues, and the route of administering GNPs into the systemic circulation avails itself to this probability. In an *in vivo* study comparing the different routes of administering GNPs and their corresponding toxicities, the authors noted that the oral and intraperitoneal routes demonstrated the highest toxicity levels, whilst the systemic route via the tail vein seemed to show the least toxicity.[151]

The GNPs used in the studies have shown PEGylation is a reliable method of protecting cells from any potential cytotoxicity from CTAB. Goodrich et al suggest that the largest accumulation of GNP *in vivo* was in the tumour followed by the liver and spleen, and the liver and spleen together accounted for approximately 75% of the injected GNPs on the first day,[140] but this gradually clears without any signs of acute toxicity throughout a 60 day period.

Discussion

The information presented here is encouraging in demonstrating that GNPs do have the potential to be excellent tumour targeting agents due to their ability to extravasate from leaky endothelial walls surrounding a GI cancer, and remain *in-situ* sufficiently long to absorb NIR light and generate heat that is capable of destroying cancer cells. Active targeting to tumours can also be accomplished by conjugation with moieties that are over-expressed on cancer cells, namely antibodies, folic acid and peptides.

Further chemical refinement of NPs is being developed, such that the cytotoxicity of these particles is becoming much less pronounced. Despite the GI studies that have been conducted, we appear to be far away from conducting a clinical trial, unless there is a concerted effort to minimise variations in synthesised GNPs and the concentrations used in *in vivo* experiments. Thus further GI theranostics research needs to focus on the challenges remaining in representing nanotechnology as a viable and safe adjunct to surgery. This focus should not solely be on proving tumour regression, but also on examining acute and long term *in vivo* toxicity, with sufficiently powered studies which assess safe and optimal GNP doses and laser fluence.

This systematic review has identified a cohort of *in vivo* studies using GI cancer cells that have been implanted and grown subcutaneously in rodents, however there have only been 2 studies[141,148] where the tumour has actually been established in an orthotopic (*in-situ*) model. Thermally ablating a superficial surface tumour with an external laser beam would present a lower risk profile than attempting the same endeavour intracorporeally with endoscopically-delivered NIR irradiation, where there would be additional factors and challenges to consider, but is vital to adequately assess and quantify that risk. In order to accomplish this, more orthotopic models of cancer should be studied with different modes of administering GNPs (intravenous, intratumoural or spraying) and laser treatment, to address the factors involved in bringing this technology to the forefront of clinical application. The depth of NIR penetration that can be efficaciously applied to a GI tumour region also needs to be quantified, so as to be certain about its applicability when given through the surface of the skin or organ. It is interesting in itself that none of the studies in this review provided any information about the absorption or attenuation co-efficients of tissues with or without GNPs, but this is a factor that limits the depth of effective NIR delivery and thus heating.

It is without doubt that the NIR wavelength provides the optimal photo absorbance for clinical application, as it can be delivered deep into tissues by avoiding absorption or scattering by tissues and endogenous chromophores such as haemoglobin or bile. To take advantage of this clinically, it is imperative to be able to fibre-optically couple the delivery of NIR laser to pre-existing endoscopic and laparoscopic instruments, such that tumours that contain functionalised GNPs can be simultaneously identified and treated by NIR irradiation. Establishing this form of optical coupling and image-guided tumour therapy should be tested as a repeatable minimally-invasive procedure. Moving nanotechnology from the bench to the clinical arena will not only diminish the overall side-effects from non-specific systemic treatment (such as chemoradiation), it may also reduce collateral damage to healthy tissues.

Some of the impetus for improving the quality of *in vivo* GNP studies should be because it is anticipated that some cancers would be detected early through fluorescence imaging acquired during endoscopic procedures. Simultaneous PTT could then be performed at the same sitting whilst under sedation, arguably avoiding the need for general anaesthesia, whilst reducing personnel requirement, hastening post-procedural recovery and facilitating earlier discharge. It would also dramatically reduce surgical time, for example in early upper or lower GI cancers, performing endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) would take the endoscopist a couple of hours, but the application of targeted laser via endoscopy would take a few seconds. This augurs well with today's enhanced recovery programmes, where there is a drive for more directed therapy, improved outcomes, reduction in soft tissue trauma, reduced complications/risks, reduced length of stay and cost-effectiveness. Nanotechnology also has the potential of being complementary to surgery, by being applicable post-operatively to tumour cavities and lymphatic tissues in order to reduce the risk of recurrence.

Equally important an issue for consideration in active targeting is tumour heterogeneity. Cancers are unique and vary from organ to organ, involving a mixture of malignant, non-malignant, stem and progenitor cells.[152] It remains to be answered if it is possible to overcome this phenomenon by utilising the optical property of GNPs. It is envisaged that this could be performed by direct intratumoural injection of GNPs into the GI cancer, and then irradiating the site with NIR. This would have a particular palliative interest, primarily being useful in patients who are unfit for surgery or those who require tumour debulking for symptomatic relief, for instance from dysphagia due to tumour ingrowth from a stented oesophageal carcinoma, vomiting and aspiration pneumonia from gastric outlet obstruction or subacute bowel obstruction from a difficult-to-stent and stenosing colorectal adenocarcinoma. Should there be sufficient grounds for a human clinical trial, it would be possible to extend this application to low volume metastatic lesions in the liver or peritoneum, whereby GNPs could be visually or ultrasonically injected intratumourally and NIR laser would then be delivered through fibres during concurrent laparoscopy. The procedure is likely to need repeating, depending on the size and location of the lesion, but it should be relatively quick to do and uncomplicated, and could potentially significantly diminish the systemic inflammatory response syndrome and the surgical risks inherent from a hemi-hepatectomy. In addition to the benefits of utilising nanotechnology in late cancers, there remain unexplored yet intriguing avenues in the theranostics of early mucosal and submucosal tumours using a combination of optimal imaging techniques and targeted PTT, which are viable research platforms for the screening and timely management of such lesions.

Conclusions

This is the first systematic review that has scrutinised the studies and collated results from the application of GNPs in the theranostics of upper and lower gastrointestinal cancer. The incorporation of a surface coating has certainly increased the biocompatibility and decreased the cytotoxicity of GNPs. Longitudinal survival studies of mice infused with varying volumes and OD of GNPs demonstrated much-needed objective confirmation that all the animals remained healthy during the study period, with evidence of prolonged survival in PTT studies. Although there appears to be an initial transient accumulation of gold chiefly in the liver and spleen after intravenous administration, this gradually dissipates sufficiently with no long-term sequelae or signs of toxicity in all *in vivo* studies.

The role of GNPs in providing diagnostic information is derived from the fact that GNPs are inherently dynamic optical contrast agents coupled with the ability to be further functionalised with NIR fluorophores which lend itself to being used in a variety of imaging techniques such as two-photon luminescence imaging, photoacoustic imaging, narrow band imaging and optical coherence tomography. This feature of optical absorption contrast and fluorescence to detect the location of GNPs within cancerous tissue would also guide the targeting of the NIR laser beam for therapy.

In terms of quantifying the efficacy of treatment on GI adenocarcinoma, all studies conducting photothermal therapy with gold nanoparticles showed cancer cell destruction and *in vivo* effects ranging from tumour volume regression to complete remission. The hyperthermia induced by laser irradiation appeared to concentrate specifically on the tumour area, with sparing of surrounding healthy tissues, enabling this technology to ultimately be a useful adjunct to surgery and be delivered in a minimally invasive way. Before such an undertaking can be realised, concordance should be reached with regards to the type, size and concentration of GNPs, with the identification of a more robust, consistent and reproducible irradiation regime. Given the evidence of their safety and efficacy, achieving this congruity would provide the final necessary credentials to establish a much-needed clinical trial of gold nanoparticles in human GI cancer theranostics.

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