Opinion TRENDS in Biotechnology Vol.25 No.4

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ScienceDirect

Applications of nanoparticles to diagnostics and therapeutics in colorectal cancer

Paolo Fortina[1,2], Larry J Kricka[3], David J Graves[4], Jason Park[3], Terry Hyslop[5,6,7], Felicia Tam[8], Naomi Halas[9], Saul Surrey[10] and Scott A. Waldman[6,7]

[1] Department of Cancer Biology, Kimmel Cancer Center, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA
[2] Dipartimento di Medicina Sperimentale, Universita' La Sapienza, 00185, Roma, Italy
[3] Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, Philadelphia, PA 19104, USA
[4] Department of Chemical and Biomolecular Engineering, University of Pennsylvania School of Engineering and Applied Science, Philadelphia, PA 19104, USA
[5] Division of Biostatistics, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA
[6] Division of Clinical Pharmacology, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA
[7] Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA
[8] Department of Physics and Astronomy, Rice University, Houston, TX 77005, USA
[9] Department of Electrical and Computer Engineering, Rice University, Houston, TX 77005, USA
[10] Cardeza Foundation for Hematologic Research, Department of Medicine, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA

Nanotechnology has considerable promise for the detection, staging and treatment of cancer. Here, we outline one such promising application: the use of nanostructures with surface-bound ligands for the targeted delivery and ablation of colorectal cancer (CRC), the third most common malignancy and the second most common cause of cancer-related mortality in the US. Normal colonic epithelial cells as well as primary CRC and metastatic tumors all express a unique surface-bound guanylyl cyclase C (GCC), which binds the diarrheagenic bacterial heat-stable peptide enterotoxin ST. This makes GCC a potential target for metastatic tumor ablation using ST-bound nanoparticles in combination with thermal ablation with near-infrared or radiofrequency energy absorption. Furthermore, the incorporation of iron or iron oxide into such structures would provide advantages for magnetic resonance imaging (MRI). Although the scenarios outlined in this article are hypothetical, they might stimulate ideas about how other cancers could be attacked using nanotechnology.

Introduction

Colorectal cancer is the third most common neoplasm and the second leading cause of cancer-related mortality in the US (Table 1) [1]. Surgery continues to have a major role in colorectal cancer survival in early-stage disease, by removing detectable tumor; however, residual micrometastases might cause a relapse [2]. Recurrence rates vary from 3% for disease limited to the mucosa (stage I) to >50% for tumors that have spread to regional lymph nodes (stage III). Overall, ~50% of surgically treated patients suffer from a relapse, with 30% recurring locally or regionally and 70% recurring at distant sites – primarily the liver and lung [3]. There is an unmet clinical need for image-based detection, targeted delivery, and ablation of metastases, to affect survival in this disease. In this context, guanylyl cyclase C (GCC), the intestinal receptor for bacterial diarrheagenic heat-stable enterotoxins (STs), which is selectively expressed in the apical membranes of intestinal mucosa cells in normal adults and in colon cancer cells, might be the 'magic bullet' for targeted ablation of colorectal cancer micrometastases [4–6].

Here, we present GCC as a receptor target in colorectal cancer, discuss diagnostics using nanostructures, and address *in vivo* imaging using iron oxide nanoparticles and near-infrared (NIR) fluorescence imaging – approaches that could be extended to other tumor types that have unique receptor functionalities. Novel therapeutic approaches based on functionalized nanoshells and iron-oxide nanoparticles for NIR and radio frequency (RF)-mediated thermal ablation, respectively, of micrometastases as well as nanostructure-based drug delivery are also evaluated. Regulatory issues related to use of nanostructures are not addressed here and have been reviewed previously [7,8].

Nanotechnology

In the past five years, the applications of nanotechnology have been realized in clinical laboratory analysis, imaging and therapeutics [9–16]. The versatility and broad applicability of nanotechnology reflect the spectra of composite materials (e.g. metals, semiconductors or polymers), geometries (e.g. sphere, prism or rod), and structures (e.g. solid, core or shell or dendrimers) that have been generated. This rapidly increasing activity is due to several

Corresponding author: Fortina, P. (paolo.fortina@jefferson.edu).
Available online 21 February 2007.

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Table 1. Causes of cancer and cancer-related mortality by site

	ESTIMATED US CANCER CASES[a]		
	Men	**Women**	
New cancers anticipated	720 280	679 510	
Prostate	33%	31%	Breast
Lung and bronchus	13%	12%	Lung and bronchus
Colon and rectum	10%	11%	Colon and rectum
Urinary bladder	6%	6%	Uterine corpus
Melanoma of skin	5%	4%	Melanoma of skin
Non-Hodgkin's lymphoma	4%	4%	Non-Hodgkin's lymphoma
Kidney	3%	3%	Thyroid
Oral cavity	3%	3%	Ovary
Leukemia	3%	2%	Urinary bladder
Pancreas	2%	2%	Pancreas
All other sites	18%	22%	All other sites
	ESTIMATED US CANCER DEATHS		
	Men	**Women**	
	291 270	273 560	
Lung and bronchus	31%	26%	Lung and bronchus
Colon and rectum	10%	15%	Breast
Prostate	9%	10%	Colon and rectum
Pancreas	6%	6%	Pancreas
Leukemia	4%	6%	Ovary
Liver and bile duct	4%	4%	Leukemia
Esophagus	4%	3%	Non-Hodgkin's lymphoma
Non-Hodgkin'slymphoma	3%	3%	Uterine corpus
Urinary bladder	3%	2%	Multiple myeloma
Kidney	3%	2%	Brain/ONS
All other sites	23%	23%	All other sites

[a]Excludes basal and squamous cell skin cancers and *in situ* carcinoma except urinary bladder. Abbreviation: ONS, other nervous systemSource: American Cancer Society, 2006 (http://www.cancer.org/docroot/PRO/content/PRO_1_1_Cancer_Statistics_2006_Presentation.asp)

factors, including: i) the discovery of new forms of matter, such as buckministerfullerenes (buckyballs) and nanotubes made of carbon [17,18]; ii) the increasing ability of materials scientists to produce reagents on a small but controlled size scale; ii) the availability of instruments, such as the atomic force microscope and the scanning tunneling microscope, to complement traditional instrumentation for viewing and characterizing nanoparticles [19]; iv) the discovery of quantum effects, such as size-dependent fluorescent emission in small particles; and v) the stimulation provided by new funding initiatives from worldwide government agencies (http://nano.cancer.gov/).

Nano-sized structures range from 1–100 nm – for comparative purposes, a single turn of the DNA helix is ~3.4 nm in length, and a eukaryotic cell has dimensions in the range 10–100 μm. Nanoparticles have been used in biology and medicine for >50 years [20]. In earlier times, they were generally called colloids, emulsions or aerosols, and included many natural and man-made suspensions. Colloidal radioactive gold or gold salts were used as therapeutic agents for intra-articular injection in patients with rheumatoid arthritis – a practice that continues in the current clinical management of this disease – and for treating cancer in the 1950s [21], and colloidal gold or gold granules were used as electron-opaque labels in electron microscopy in the 1960s.

Some of the newer techniques for producing nanoparticles involve: i) cooling them from a hot gas or plasma; ii) reacting a gaseous mixture at a hot surface; iii) designing a system with defined properties so it forms a geometrically precise multi-phase structure as it solidifies; and iv) forming by self-assembly, in which components are designed so that intermolecular forces cause them to come together in a predetermined fashion. Multi-step procedures can be used to produce sophisticated core and shell structures that can be further engineered to have highly controlled and 'tunable' properties. These include paramagnetic nanoparticles, which can serve as contrast agents for the MRI of tumors [22–24], and nanoshells, which can be fabricated with defined metal shell thicknesses to absorb specific wavelengths of NIR light, resulting in their plasmon resonance and transfer of thermal energy to the surrounding environment [25,26]. In this latter example, NIR wavelengths have minimal optical absorption and, consequently, optimum penetration in tissues, resulting in minimal thermal injury to structures that lack the nanoparticles.

Furthermore, nanostructures can be conjugated to biological molecules, including hormones and antibodies, which enables their targeting to tissues expressing their cognate receptors [27–29]. For example, fluorescent quantum dots conjugated to various peptides specifically target either the vasculature of normal tissues or, alternatively, of cancer cells [30]. Tumor-specific monoclonal antibodies can be conjugated to nanostructures. Monoclonal antibodies directed against a prostate-specific membrane antigen (PSMA) were attached to the surface of triblock copolymer-modified quantum dots and used to image prostate cancer cells, both *in vitro* and *in vivo*, in tumor xenografts [31]. Indeed, anti-PSMA quantum dots injected IV and visualized fluorescently by macro-illumination accumulate in and are retained by prostate cancer xenografts in nude mice.

Nanoshells

Nanoparticles composed of metallic shells with dielectric cores have a tunable plasmon resonance based on the

relative dimensions of the dielectric core and metallic shell [23–26]. Nanoshells composed of metallic gold coating a dielectric core, such as silica, resonate at specific wavelengths of light ranging from 500 nm to 2 μm, depending on their core:shell thickness ratio. Indeed, gold shells of 10 nm encasing a 110 nm silica core resonate in the NIR spectrum (~800 nm); these wavelengths exhibit minimal optical absorption by, and consequently optimum penetration through, overlying tissues, with minimal attendant thermal injury (Figure 1). In the context of specifically absorbing energy in the NIR spectrum, these nanoshells are extremely efficient in converting optical energy into heat. When exposed to NIR light, nanoshells with a 10 nm gold shell and a 110 nm silica core induce thermal damage in adjacent cells [25]. Similarly, direct injection of nanoshells into subcutaneous tumors in a mouse xenograft model resulted in temperature increases of ~37 °C greater than the surrounding normal tissue after ~6 min of exposure to 808 nm at 4 Watts/cm^2, associated with thermal damage to tumors [26]. Separately, nanoshells injected IV into mice with subcutaneous tumors resulted in NIR heating of tumors compared with non-tumor sites and control animals; most probably, this damage resulted from the enhanced permeability of, and retention by, the tumor neovasculature compared with the established vasculature in normal tissues [26]. Differential heating was associated with complete regression of subcutaneous tumor xenografts in animals receiving gold nanoshells compared with control animals. Nanoshells that specifically target tumor cell receptors might be expected to perform even better, particularly on larger and more mature tumors.

Targeting nanoshells and iron-oxide nanoparticles for receptor-directed thermal ablation

The realization of the full diagnostic and therapeutic potential of nanoparticles *in vivo*, in part, reflects the ability to target their localization and uptake to specific cells and tissues. Initial studies with nanoshells for thermal ablation depended on preferential delivery to tumors by exploiting the increased permeability and retention within the tumor neovasculature [32,33]. However, the gold surface of nanoshells can be readily conjugated to biologically active molecules, including antibodies [34]. Thus, an ortho-pyridyl-disulfide-*N*-hydroxysuccinimide polyethylene glycol polymer was used to conjugate HER2-specific IgG antibodies to gold nanoshell surfaces. Under conditions of dark-field microscopy, binding of the anti-HER2 nanoshells to HER2-positive SKBr3 human mammary adenocarcinoma cells was detected by gold-specific silver enhancement staining. These observations highlight the ability of gold nanoshells that are suitable for thermal ablation to be conjugated to targeting molecules for tumor-specific delivery. Iron-oxide or iron-cored nanoshells should provide contrast agents suitable for MRI imaging, which, in addition to their non-invasive nature, would provide even more powerful and generally useful diagnostic tools [14].

Nanometer-sized particles of different shapes and compositions are emerging as important new tools for cancer

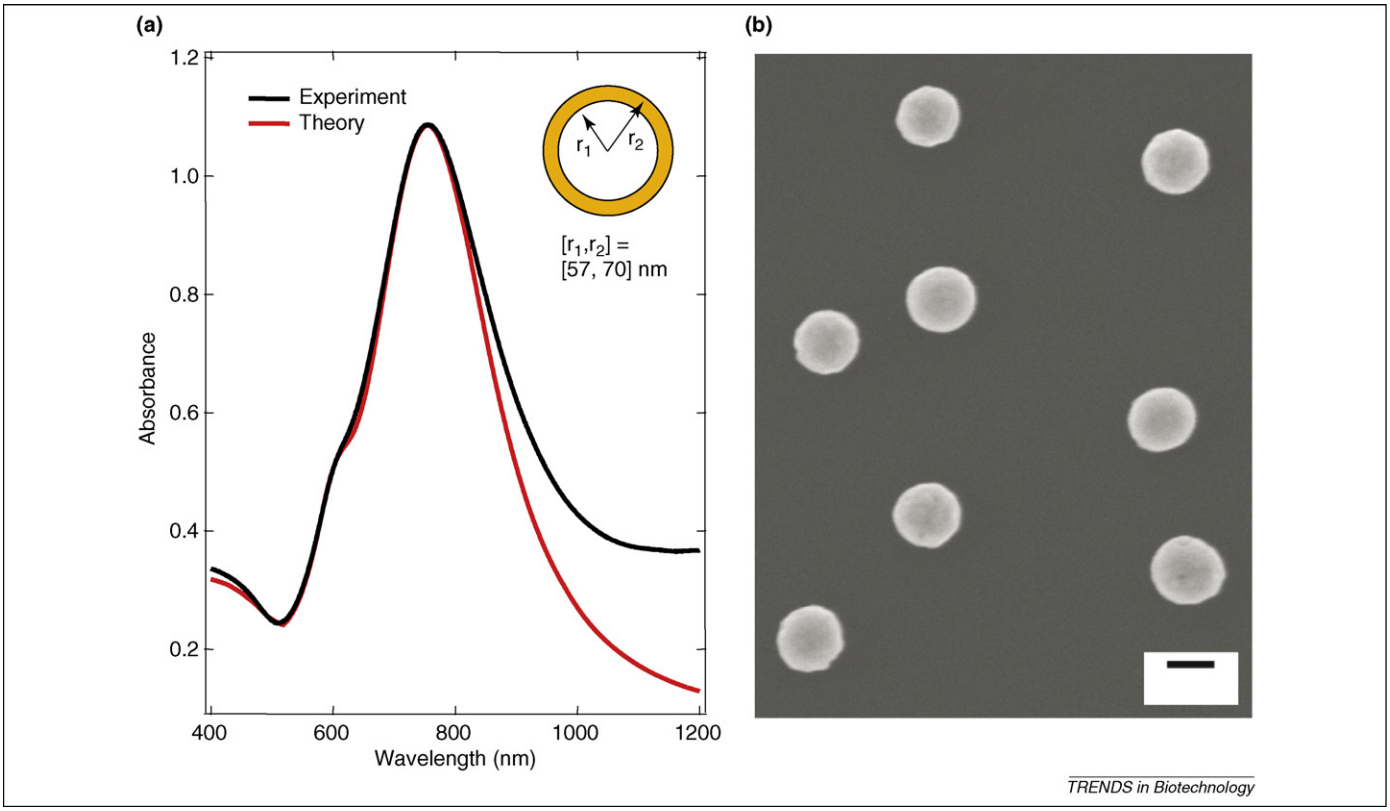

Figure 1. Gold–silica nanoshells tuned to NIR wavelengths. Gold nanoshells are synthesized using SiO_2 cores (~114 nm diameter) with surface seeding using 1–3 nm gold particles on cores, followed by controlled surface fill-in with gold by reductive deposition from a gold salt solution. **(a)** The experimental and theoretical extinction spectra of nanoshells with [r_1, r_2] = [57,70] nm (inset). The radii define the inner and outer diameters of the shell and hence its thickness, leading to the generation of gold nanoshells with an absorption maximum in the NIR (~780 nm). **(b)** Scanning electron micrograph of synthesized gold nanoshells. Bar = 100 nm.

diagnosis, staging and therapeutics. The lack of broader adoption might reflect the considerable barriers to implementation (e.g. availability of particles and suitable automated detection systems) and the absence of biosafety data [35]. Potential applications of thermal-induced tumor ablation by targeted delivery of gold nanoshells to colon cancer cells and, by analogy, to other types of tumors with unique receptors will be addressed below.

Colorectal cancer

Guanylyl cyclase C

GCC is the intestinal receptor for the high-affinity, bacterial, diarrheagenic, heat-stable enterotoxins (STs) and the lower affinity endogenous ligands guanylin and uroguanylin, which induce accumulation of cGMP [4]. In turn, cGMP activates a protein kinase that phosphorylates ion channels, mediating the efflux of salt and water and, in the case of ST, causes diarrhea (Figure 2). GCC is selectively expressed in the apical membranes of intestinal mucosa cells and colon cancer cells. Indeed, expression of GCC has been detected in all colorectal tumors, but not in extragastrointestinal tissues or tumors [4,36] Selective overexpression of GCC by colorectal cancer cells and its anatomical compartmentalization – it is normally confined to mucosa but is accessible to the systemic vasculature in metastatic disease – suggests its utility as a target for delivering therapeutic agents *in vivo*.

GCC as a specific receptor target in colon cancer cells

GCC might be uniquely suited for targeting novel therapeutic agents to metastatic colorectal cancer cells [36]. More specifically, if nanoshells of defined optical resonance frequencies or RF absorptivity can be conjugated to ST for specific delivery, targeted by GCC, it might prove possible to thermally ablate metastatic colorectal tumors. The use of infrared energy in a band where there is minimal absorption by normal tissues is attractive and has been explored experimentally in animals. Unfortunately, thicker tissue masses, such as those found in humans, and highly vascularized (IR absorbing) tissue, such as the liver, present potential problems. RF heating is an alternative with some experimental foundation. It could prove useful, particularly if MRI imaging is used first to locate metastases for focused energy application.

Conjugation of ST to organic and inorganic molecules preserves ligand binding

The 18 amino acid core peptide of ST is sufficient for GCC receptor binding with nanomolar affinity and full biological activity, *in vitro* and *in vivo*. Although manipulation of the ST carboxyl terminus eliminates receptor binding and biological activity, manipulation of the N-terminus preserves binding and enables delivery of large, conjugated, heterologous molecules to colorectal cancer cells. ^{125}I-ST conjugated to biotin, using a long-chain hydrocarbon linker arm coupled to *N*-hydroxy-succinimide, was found to bind to receptors in the GCC positive T84 cell membranes in a time- and concentration-dependent fashion. Binding was also quantitatively competed out by unlabeled ST, demonstrating specificity of the receptor interaction [36]. Analyses of equilibrium binding by the method of Scatchard demonstrated curvilinear isotherms, and the binding parameters derived from those analyses were comparable to those obtained with unconjugated ^{125}I-ST. In addition, labeled ST conjugated to biotin and avidin was specifically internalized in a time-dependent fashion by T84 cells [37,38]. Similarly, biotinylated ST was immobilized on streptavidin or anti-biotin antibody coated polyvinylchloride plates and used to capture cells expressing ST receptors, with high affinity and specificity [39]. Furthermore, biotinylated ST was coupled to streptavidin–gold and used to label cells expressing GCC, with high affinity and specificity. These data demonstrate that ST can be conjugated to molecules with dimensions and compositions comparable to gold nanoshells and retain full receptor-binding function and the ability to undergo internalization.

Hence, ST attached to gold nanoshells, when injected, should target to micrometastases and/or tumor sites. ST-binding selectively occurs through surface-bound GCC on cancer cells followed by ST-bound nanoshell internalization. NIR radiation could then be used, and the energy absorbed by the internalized gold nanoshells results in heat emission and thermal ablation of the micrometastases. Alternatively, RF-induced ablation of targeted iron-oxide particles could be used; studies are underway using cell lines and animal models of colon cancer to validate such an approach.

Colon cancer diagnostics and therapeutics using nanostructures

The application of nanotechnology to the diagnosis and treatment of colorectal cancer has the promise of enhancing conventional methods as well as fostering the development of novel approaches for detection and therapy. With respect to diagnostics, assays can be divided into *in vitro* (e.g. diagnostic tests on blood serum) and *in vivo* (e.g. imaging of administered agents) applications. Expected improvements for *in vitro* diagnostics include increases in analytical sensitivity without sacrificing specificity. The expected benefits of improved analytical performance include non-amplification assays that are faster and require smaller sample sizes and less expensive detection technologies, which can be readily miniaturized. These benefits might result in greater accessibility of diagnostic tools to clinicians and patients. With regard to *in vivo* imaging, enhancement of conventional imaging agents has resulted in higher sensitivity and finer resolution of tumors. Furthermore, the application of nanotechnology to develop novel imaging agents has resulted in new roles for non-invasive imaging in the detection, staging and overall management of patients with cancer [10–16]. With respect to therapy, targeted nanostructures offer potential solutions to the limitations of standard chemo- and radiotherapeutic modalities, particularly in the context of collateral damage to normal tissues in proximity to, and distant from, tumors and the associated dose-limiting clinical toxicities, which severely restrict the efficacy of the current therapeutic armamentarium.

Although there are many examples of the application of nanotechnology to various analyses and diseases, there are few examples that directly address the *in vitro* diagnosis of

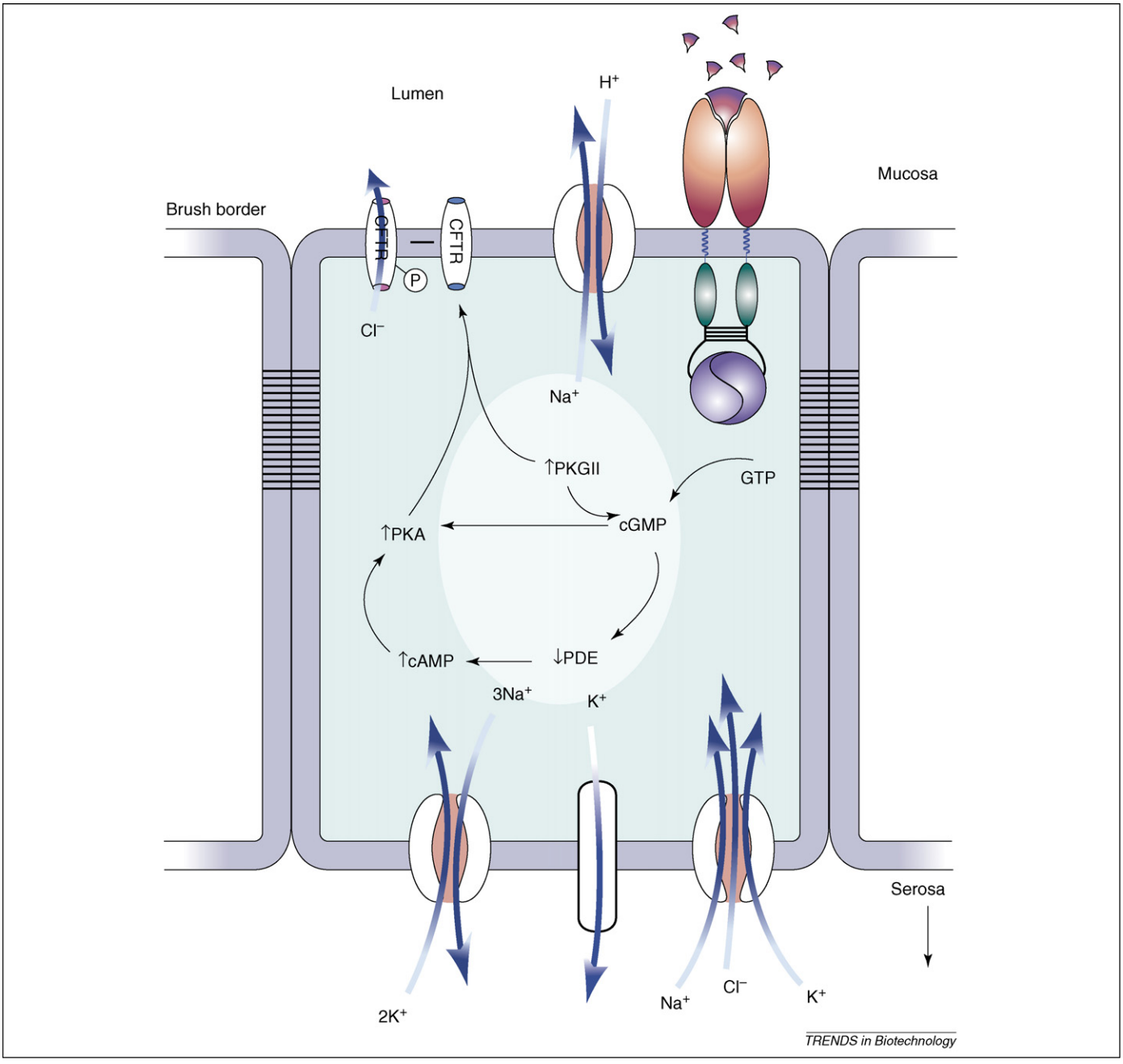

Figure 2. Regulation of intestinal secretion by the heat-stable enterotoxin (ST) and GCC. Bacteria, such as *E. coli*, containing plasmids that encode a member of the homologous peptide family of STs colonize the intestine following the consumption of contaminated food and/or water. Bacterial colonization leads to production of ST in the gut lumen. ST specifically recognizes and binds to the extracellular domain of GCC, which is expressed in the brush border membranes of intestinal mucosa cells from the duodenum to the rectum. The interaction of ST and the extracellular domain of GCC is translated across the plasma membrane to activate the cytoplasmic catalytic domain, resulting in the production and accumulation of cGMP. This cyclic nucleotide binds to and activates cGMP-dependent protein kinase II (PKG II), also localized in the intestinal cell brush border membrane. Furthermore, cGMP can activate cAMP-dependent protein kinase (PKA), either directly or by inhibiting a cAMP-specific phosphodiesterase (PDE), and induces the accumulation of cAMP. The cystic fibrosis transmembrane conductance regulator (CFTR), which is co-localized with GCC and PKG II in brush border membranes, is a substrate for this protein kinase and PKA. CFTR is a chloride channel, and its phosphorylation by PKA or PKG results in a persistent open state, permitting chloride to flow down its concentration gradient from the intracellular to the extracellular compartment. Other ion channels and transporters in the cell maintain the electroneutrality of ST-induced chloride efflux. Vectoral water flux from the basolateral to the apical surface is driven by these ionic conductances, resulting in the accumulation of fluid and electrolytes in the intestinal lumen and secretory diarrhea.

CRC. However, the potential of nanostructures is apparent from the results from other disease systems. Nanostructures, particularly nanoparticles, offer a diverse range of labels that provide direct visual detection and multiplexing capabilities for assaying proteins or detecting nucleic acids [40]. Some of the many types of nanostructures that have been used as components of *in vitro* diagnostic tests for protein markers or nucleic acid targets are presented in Table 2, and a comprehensive bibliography is available from the National Cancer Institute (http://nano.cancer.gov/resource_center/scientific_bibliography.asp). These nanostructure-based analytical strategies could also be applied to the analysis of specific colon cancer-related markers or nucleic acid sequences.

Similar to *in vitro* diagnostics, the use of nanostructures for *in vivo* imaging is also a particularly promising area of

Table 2. Nanostructures for diagnostic applications

In vitro diagnostic		
Nanostructure	**Application**	**Refs**
Nanochannel		
Glass	DNA sequencing	[a]
Nanocrystal		
CdS, CuS, PbS	Single-nucleotide polymorphism	[48]
Fluorescein diacetate	IgG	[49]
Nanoparticle		
EuIII-chelate-doped polystyrene	PSA	[50]
Au	Prion protein	[51]
2-methacryloyloxyethyl phosphorylcholine	C-reactive protein	[52]
Polystyrene	Single-base mutation	[53]
Silica	Calf thymus DNA	[54]
Ag on Au	IgG	[55]
Tris (2,2′-bipyridyl) dichloroRu (II) hexahydrate-doped silica	IgG, DNA	[56]
Nanopore		
Silicon nitride	DNA sequencing	[57]
Nanoprism		
Ag	–	[58]
Au	–	[59]
Nanorod		
Au/Ag/Ni/Pd/Pt	IgG	[60]
Nanotube		
Carbon	DNA	[61]
Nanowire		
Si	Influenza A	[62]
Au	*E. coli*	[63]
Polypyrrole	DNA	[64]
***In vivo* diagnostic**		
Nanostructure	**Application**	**Refs**
Liposome		
Gadolinium	MRI imaging	[65,66]
Dual-fluorescence or iron oxide	Optical and MRI imaging	[67]
Dendrimer		
Gadolinium	MRI imaging	[41,42]
Nanoparticle		
Dextran-coated iron oxide	MRI imaging	[45]
Quantum dots	Near-infrared imaging	[46,47,68]
Gold	Optical detection	[69]
Nanoshell		
Gold	Optical detection	[70]
Nanotube		
Ultrashort Gd packed nanotubes	MRI imaging	[71]

[a] http://www.ece.cmu.edu/~mems/pubs/show.php?pub_id=160

colon cancer diagnostics. However, there are few examples of the application of nanotechnology to the imaging of CRC. Several examples that have been applied to enhancing detection and management of various diseases are outlined in Table 2. The technology most readily applicable to colorectal cancer includes novel contrast agents for MRI. Nanostructures that modify conventional contrast agents, such as gadolinium, or imaging agents, such as iron oxide, have the potential to enhance the diagnostic power of clinical imaging [41–45]. Not only do these nanostructures improve the features of conventional MRI imaging, they also present opportunities to change how colorectal cancer is detected and managed.

Among the technologies in development that might result in the introduction of new modalities in CRC imaging is the use of near-infrared fluorescence imaging (NIRF). NIRF can be useful for imaging gastrointestinal diseases such as CRC because the current clinical evaluation of CRC already uses fiber optic examination of luminal surfaces [46]. This standard of practice can be enhanced by endoscopic visualization of near-infrared fluorescing imaging agents such as tunable quantum dots. Indeed, a murine model of colon cancer has already been studied using a NIRF agent [47].

Beyond imaging and detection, targeted nanostructures offer opportunities to develop novel approaches to treat colorectal and other tumors [10]. Liposomal encapsulation of anthracyclins, including doxorubicin, has had success as an FDA-approved treatment for metastatic ovarian cancer refractory to paclitaxel- and platinum-based agents. Moreover, these stealth nanostructures have been investigated for the treatment of breast cancer, non-Hodgkin's lymphoma and small-cell lung cancer. Similarly, nanoparticle delivery of paclitaxel is being used for the treatment of patients with metastatic breast cancer who have failed standard adjuvant chemotherapy, including anthracycline-based regimens. In preclinical development, nanostructures that are targeted specifically to tumor-expressed molecules are being used to improve the specificity of delivery of cytotoxic chemotherapeutics and limit the collateral damage to normal tissues that is normally associated with their use. Indeed, nanostructures have been used in conjunction with antibodies, proteins and small-molecule ligands targeted to specific tumor-associated receptors to deliver chemotherapeutic agents. This results in greater pharmacological and clinical efficacy and is associated with lower adverse events in animal tumor-xenograft models. In this context, antibody-targeted liposomes effectively accumulate in colorectal cancer cells in mouse xenograft models. These next generation of nanostructured delivery systems, with demonstrated efficacy in animal models, will be translated into early-phase clinical trials in the near-term for the delivery of chemotherapy to cancer patients.

Conclusion

The broad field of cancer management has a history of quickly replacing existing methodologies with ones that offer advantages in terms of improved diagnostic capabilities, increased sensitivity for tumor staging, and better therapeutic approaches and drug delivery. Nanotechnology has several beneficial attributes that might improve the management of colon cancer patients. Indeed, there are now signs that the potential provided by nanotechnology is being evaluated, and the first steps are being taken in the development of improved colorectal cancer imaging, therapeutics and the eventual implementation of targeted

strategies for ablation. Although this Opinion has focused on CRC as a particularly tempting target for the early application of nanotechnology-based approaches, the potential for other tumors with similar highly specific target molecules should be clear.

Acknowledgements

This work was supported, in part, by grants from the PA Department of Health (SAP4100026302 to PF), NIH (HL-69256 to SS, CA75123, CA79663 and CA95026 to SAW), Targeted Diagnostic and Therapeutics, Inc., West Chester, PA (to SAW), the Cardeza Foundation for Hematological Research, Philadelphia, PA (to SS), the Kimmel Cancer Center, Philadelphia, PA (to PF) and the Robert A. Welch Foundation C-1220, the Department of Defense Breast Cancer Research Program (DAMD17-03-1-0384 to NH and FT) and the Texas Institute for Bio-Nano Materials and Structures for Aerospace Vehicles funded by NASA (NH, FT). SAW is the Samuel M.V. Hamilton Endowed Professor.

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