Role of Raman spectroscopy and surface enhanced Raman spectroscopy in colorectal cancer

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Abstract
Colorectal cancer (CRC) is the fourth most common cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting. Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) are forms of vibrational spectroscopy that offer a non-destructive method to gain molecular information about biological samples. The techniques offer a wide range of applications from in vivo or in vitro diagnostics using endoscopic probes, to the use of micro-spectrometers for analysis of biofluids. The techniques have the potential to detect molecular changes prior to any morphological changes occurring in the tissue and therefore could offer many possibilities to aid the detection of CRC. The purpose of this review is to look at the current state of diagnostic technology in the United Kingdom. The development of Raman spectroscopy and SERS in clinical applications relation for CRC will then be discussed. Finally, future areas of research of Raman/SERS as a clinical tool for the diagnosis of CRC are also discussed.

Key words: Detection; Colorectal cancer; Spectroscopy; Raman; Surface enhanced Raman spectroscopy

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Core tip: This review focuses of the current role of Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) in clinical applications of colorectal cancer. This includes a review of the current research into in vivo endoscopic Raman probes, non-destructive analysis of biofluids and the use of SERS in order to detect low concentration analytes that previously could not be detected with Raman spectroscopy. Both the advantages and disadvantages of the technology are discussed along with possible avenues of future research.
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INTRODUCTION
Colorectal cancer (CRC) is the fourth most common cancer in the United Kingdom and is the second largest cause of cancer related death in the United Kingdom after lung cancer[1]. Currently in the United Kingdom there is not a diagnostic test that has sufficient differentiation between patients with cancer and those without cancer so the current referral system relies on symptomatic presentation in a primary care setting[2,3]. CRC results from the progressive accumulation of genetic and epigenetic alterations that disrupt normal cellular mechanisms[4]. The 5-year survival rate for CRC detected in early stages are > 90%, however the 5-year survival rate for later-stage cancers is < 10%[5,6]. This highlights the need for a simple, reliable diagnostic test that can detect early signs of the disease. The majority of CRCs present symptomatically in a primary care setting so it is important that general practitioners can identify patients who are at highest risk[3]. The risk associated to patients is based on the referral guideline, in the United Kingdom is this based on a combination of symptoms and age of the patient[6]. The relationship between initial symptoms and mortality as a diagnostic indicator have previously been discussed in depth[2,3,7,8]. Unfortunately initial symptoms associated with CRC can also be symptoms of benign diseases such as irritable bowel syndrome[9], and there is currently no diagnostic test available in primary care that has sufficient differentiation to base referral on[3]. Furthermore the detection of CRC using symptoms has been shown to be ineffective for decreasing mortality rates in comparison to the European average[9].

Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) could hold many advantages for use as a diagnostic tool for CRC. These techniques have previously been used to discriminate between cancerous and non-cancerous tissue, biofluid samples, as well as in the development of in vivo Raman systems for use in endoscopy. The techniques are non-destructive to samples so can provide molecular information about a sample without the need for staining or in some cases without the need for resection. Raman and SERS have the potential to detect molecular changes in cancerous cells/tissues and biofluids that precede morphological changes such as the development of precursor lesions. The techniques could offer the potential for an early detection tool that detects molecular changes before the stage at which a traditional histopathology would be able to detect. This review paper will discuss the previous applications of Raman spectroscopy and SERS to detect CRC.

CURRENT DIAGNOSTIC PATHWAYS
In United Kingdom, CRC survival outcomes are lower than the European average, this is be attributed to a higher proportion of cancer being diagnosed at late-stage[9]. In order to combat the NHS rolled out a national screening program in 2006. There are currently two types of screening method available to screen for CRC and late-stage adenoma namely flexible sigmoidoscopy (FS) and measurement of markers in faecal samples[10].

FS is a procedure involving the insertion of an endoscope into the rectum of a patient in order to examine the distal colon and rectum; the procedure must be carried out by a trained doctor. Atkin et al[11](2010) conducted a United Kingdom study into the effectiveness of a single FS procedure as a screening tool. A procedure for patients aged 55-64 years the study saw a 33% reduction in CRC cases and a 43% mortality reduction, however this is only applicable with respect to the distal colon[11]. However, the cost of a FS is high in comparison to tests that look for markers in faecal samples because it involves trained staff[12]. Moreover, it can be argued that the results of tests reliant on the opinion of a practitioner can be subjective depending on the experience of the practitioner.

In England and Wales the guaiac faecal occult blood test (gFOBT) is commonly used as a screening tool. The test is simple and cheap compared to other methods[12]. Problems that occur with gFOBT regarding dietary requirements of patients before taking the test are solved using immunochemical faecal occult blood testing (iFOBT)[13]. There are different types of iFOBT, all of which detect human specific haemoglobin in faecal samples. Investigations and meta-analysis studies have shown iFOBT to have improved sensitivity in the detection of CRCs and late-stage adenomas in both high-risk, average-risk and populations with no overt rectal bleeding compared to gFOBT without compromising on specificity[14-17]. However, despite the improved sensitivity of iFOBT tests patient uptake of the screening programme in general is poor. In October 2008 of the 2.1 million people that had been invited to partake in the screening programs in England uptake was just 55%-60%[18]. Other diagnostic methods available to clinicians in the United Kingdom include double barium enema, computed tomography colonoscopy, MRI imaging and colonoscopy. Outside of the United Kingdom there are other diagnostic tests are also in use such as the tumour M2-pyruvate kinase (tumour M2-PK) test. This is a faecal test that investigations have shown this to also be more sensitive than FOBT[19]. However, the cost effectiveness and the sensitivity and specificity of the M2-PK still needs to be established in the United Kingdom as published results are in disagreement as to whether it is more effective than iFOBT[20]. There is also a real time polymerase chain reaction based blood test that is available outside of the United Kingdom that detects methylated Septin 9 (mSept9). The blood test...
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has been shown to have sensitivity and specificity ranging from 50%-90% and 88%-91% respectively\[^{21}\]. A blood test is a potentially more attractive option for patients compared to faecal and colonoscopy tests so studies are underway to determine if the higher cost of mSept9 would be recovered by higher screening uptake\[^{22}\].

Raman spectroscopy could offer a highly sensitive and less invasive alternative/complimentary technique to aid CRC detection. However, Raman spectroscopy has not yet found its way into a routine clinical setting. This review will examine the current research status of the application of Raman spectroscopy for detecting CRC to critique the prospective translation of the technique to a clinical setting.

**Methods for review**

A systematic literature search (PubMed, MEDLINE, Web of Science) was conducted using the following terms: “Diagnosis”, “Raman spectroscopy”, “Surface enhanced Raman spectroscopy”, “SERS”, “CRC”, “Tissue”, “Biofluids”, “Immunoassay”, “CEA”. Following these searches 30 studies were selected for inclusion based on the criteria: (1) That they are original studies based on the clinical applications of Raman spectroscopy, SERS or both for CRC detection in human tissue, biofluids or cell lines; and (2) in the case where there are large amounts of similar studies that they were the first to report such data. Only studies published up to January 2015 were included in this review.

**RAMAN SPECTROSCOPY AND SERS FOR THE DETECTION OF CRC**

**Introduction**

Raman spectroscopy is a type of vibrational spectroscopy that allows the user to gain molecular information about a sample through the scattering of incident light. In general, when light is passed through or onto a sample a small proportion of the photons are scattered (approximately 10^{−5}). The majority of this is Rayleigh or elastic scattering; where the energy of the incoming photon is equal to the energy of the scattered photon (Figure 1)\[^{23}\]. Around 1 in 10^{7} of the incident photons are in-elastically scattered resulting in the incident photon and the scattered photon having a difference in energy.

The inelastic scattering is a relatively weak effect which was first observed in 1928 by Sir CV Raman and is known as Raman scattering\[^{24}\]. When scattered light is measured with a spectrometer a series of lines are observed, the shift in the energy [measured by wave-number (cm^{−1})] from the Rayleigh line (equal to incident energy) is known as the Raman shift. The shift recorded corresponds to specific vibrational or rotational modes of the sample molecule. The intensity of the “Raman shift” for a particular molecule is directly proportional to the concentration of that molecule within a sample so the resulting spectrum of a sample will be a superposition of Raman response of all the Raman active molecules from within a sample, e.g., proteins, nucleic acids, etc. It is interpreted as the molecular “fingerprint” of the sample, an example of a spectrum can be seen in Figure 2. It is due to the “fingerprint” that Raman is seen to be desirable for application to cancer detection because it offers the possibility of detecting minute differences in analyte concentrations and is a non-destructive technique.

**Vibrational techniques**

It should be noted that Raman is not the only type of vibrational spectroscopy that has been reviewed for clinical applications\[^{25,26}\]. One of the other main areas of vibrational spectroscopy being applied to clinical applications is fourier transform infrared spectroscopy (FTIR) and a type of enhancement infrared attenuated total reflection. FTIR relies on either the absorbance or the transmission of light through a sample, then similar to Raman the difference in the emitted and absorbed or transmitted light is measured to gain molecular information about a sample. This technique has also been used for diagnosis of CRC independently and also coupled with immunohistochemical staining\[^{27,28}\].

Compared with other vibrational spectroscopy techniques Raman holds many desirable properties for the application to a screening method. One of the biggest advantages of using Raman spectroscopy is that samples can be in aqueous solutions due to water having a small Raman cross-section at near-infrared wavelengths, water has a high absorbance in FTIR and therefore can interfere with a spectrum. Table 1 shows a comparison table constructed from literature to give an overview of the strengths and weaknesses of FTIR, Raman and traditional hematoxylin and eosin staining (H and E) when used for clinical applications\[^{25,26,30}\]. It suggests that for the application to biological samples such as tissues and biofluids Raman could be the most favorable option. Like FTIR it is non destructive and can be performed in real time compared to H and E staining. However, Raman spectroscopy has the advantage over FTIR of being able to scan over a larger wavenumber range than FTIR and with better spatial resolution than both FTIR and H and E. Furthermore, Raman is a technique that relies on scattering so measurements can be taken with single ended endoscopic probes. This is advantageous for in vivo applications because it makes it possible to study
samples that are optically too thick for transmission techniques. This, along with the need for a less invasive diagnostic tool that can analyse liquid biofluids leads to the remainder of this review focusing on the application of Raman and SERS technology.

**Data manipulation**

Many clinical applications of Raman and SERS use chemometric data analysis techniques in order to aid data manipulation and to pick out the small differences that could indicate disease. Details of principle component regression (PCR) and partial least squares can be found in Kramer (1998). Principle component analysis (PCA) applications can be found in Shinzawa et al. (2009). This review will only state the technique that has been used in each study.

**CLINICAL APPLICATIONS OF RAMAN SPECTROSCOPY IN CRC**

Histopathological analysis of tissue biopsies is still considered the gold standard for the diagnosis of malignant tissues that have been surgically resected. Typically, thin sections of tissue are “fixed” usually using formalin and then mounted onto glass slides and stained using various methods in order to determine TNM stage, tumour type, histologic grade and the level of vascular invasion. However, histopathology is a slow process that requires a trained pathologist, it is also inherently subjective.

Raman spectroscopy offers the possibility of determining the presence of malignancy by detecting differences in Raman spectral features between normal and malignant tissue. Previously, Raman spectroscopy has been applied to in vivo probes that have the ability to discriminate multiple tissue types, biofluid analysis and also analysis of cancerous cell lines for both discrimination and characterization. The motivation behind using Raman used in vivo is to aid rapid diagnosis and help to identify possible areas of tissue for biopsy that might otherwise be missed. A summary of the literature and different applications of Raman towards clinical applications for CRC can be found in Table 2.
Table 2  A summary of the different clinical applications of Raman spectroscopy to colorectal cancer

| Method          | Sampling type | Sample number | Ref.          | Year | Spectral region (cm\(^{-1}\)) | Laser excitation (nm) | Data analysis |
|-----------------|---------------|---------------|---------------|------|-------------------------------|-----------------------|---------------|
| Probe           | In vivo (tissue) | 20            | Shim et al\(^{42}\) | 2000 | 450-1800                      | 785                   | PCA, PLS, ANN |
| Probe           | In vivo and ex vivo (tissue) | 9            | Molckovsky et al\(^{44}\) | 2003 | 900-1800                      | 785                   | PCA, LDA, LOOCV |
| Micro-spectrometer | In vivo (primary tissue) | 10          | Chen et al\(^{45}\) | 2006 | 500-1900                      | 782.5                 | PCA           |
| Probe           | Ex vivo (tissue) | 59            | Widjaja et al\(^{43}\) | 2008 | 800-1800                      | 785                   | PCA, LDA, LOOCV |
| Micro-spectrometer | Ex vivo (tissue) | 54            | Beljebbar et al\(^{46}\) | 2009 | 600-1800                      | 785                   | SVM, PCA, LDA |
| Micro-spectrometer | In vitro (serum) | 120           | Li et al\(^{47}\) | 2012 | 800-1800                      | 785                   | PCA, PLSR, LOOCV |
| Micro-spectrometer | In vitro (cell lines) | N/A | Ranc et al\(^{48}\) | 2013 | 400-1800                      | 532                   | PCA           |
| Probe           | In vivo (tissue) | 177           | Wood et al\(^{49}\) | 2014 | 800-1800                      | 830                   | PCA, LDA, LOOCV |
| Micro-spectrometer | In vivo (tissue) | 50            | Bergholt et al\(^{50}\) | 2014 | 800-1800 and 2900-3600        | 785                   | PLS, LDA      |

PCA: Principle component analysis; LDA: Linear differential analysis; PLS/PLSR: Principle least squares regression analysis; LOOCV: Leave one out cross validation; ANN: Artificial neural network; SVM: Support vector machine; PCR: Principle component regression.

Table 2 shows that most clinical applications of Raman have been in the near-infrared (NIR) region using 785 nm laser excitation analysing tissue samples. This is likely due to reduced fluorescence of biological; samples in the NIR region, furthermore 785 nm laser is less powerful than visible region lasers so is less likely to cause damage to biological samples. The work is generally done in the “fingerprint region” of the Raman spectrum, i.e., 400-1800 cm\(^{-1}\) due to molecular bonds present in biological samples being Raman active in this region. All of the studies use chemometric data analysis so it seems for NR this is essential to differentiate the small differences in the Raman spectra when using biological samples. It is also clear that work in the field has previously been dominated work towards in vivo Raman probes for use during endoscopy but more recent work has used both Raman probes and microspectrometers.

Tissue analysis

Reviews dedicated to Raman spectroscopy for the use of in vivo probes for clinical use for many types of tissue including tissues of the gastrointestinal (GI) tract are already available\(^{25,40,41}\). Briefly, the in vivo probes for use during endoscopy for use with GI tissue was first introduced by Shim et al\(^{42}\) (2000). In general, the in vivo probes use a laser excitation source in the NIR wavelength range (light is non-mutagenic in this region) coupled to an optical fibre probe. The probe can then act as both a source of light and a detector relaying a signal back to a charged coupled device detector and computer analysis. The development of the probes is not a simple process, some materials that the probes are manufactured from have a large Raman cross sections leading to design challenges regarding signal to noise ratio (gaining unwanted noise from the material). Other issues can be caused by tissue fluorescence signal being larger than the Raman signal making data acquisition difficult and spectral acquisition times to be impractical for clinical applications (more than 10 min). Nevertheless, some research groups have been successful in designing probes for specific use with gastrointestinal tissue for use in routine endoscopy that have short acquisition times\(^{35,43}\).

Shim et al\(^{42}\) (2000) successfully applied an in vivo probe to gain Raman spectra of colonic and oesophageal tissues from 20 patients. The pressure spectral acquisitions were made with 5 s exposure time and repeated at normal and malignant sites within both areas of interest. In the colonic tissue subtle differences between spectral area 1100-1800 cm\(^{-1}\) were identified however no significant prominent changes were evident. PCA and LDA analysis was then introduced in order to try and distinguish accuracy for application to GI diagnostics however no specific results relating to diagnostics were published.

Molckovsky et al\(^{44}\) (2003) were the first to assess the diagnostic potential of near infrared Raman spectroscopy on colonic tissue by using adenomatous polyps as a model for dysplasia. The group used a custom-made fiber-optic Raman probe and used PCA-LDA analysis and LOOCV to analyse a total of 33 polyps from 8 patients. After an initial ex vivo study of polypsectomy specimens that involved a total of 54 spectra the analysis algorithm identified a sensitivity of 91% and a specificity of 95%. An in vivo study was then conducted with a total of 19 spectra form 9 polyps, after spectral analysis the algorithm identified adenomas with a sensitivity of 100% and specificity of 89%. A similar study involving a higher number of specimens for a similar use on ex vivo tissues was conducted by Widjaja et al\(^{43}\) (2008), the group were able to differentiate cancerous tissue with 100% sensitivity and 98.1%-99.7% specificity using a diagnostic algorithm using PCA and LDA.

Raman spectroscopy has also been investigated as a complimentary technique to histopathology. The first application of Raman spectroscopy for characterisation of colonic tissue (among others) to discriminate between cancerous vs normal was by Feld et al\(^{46}\) (1995). The group looked at the difference spectra between normal and cancerous tissue, this results showed potential that spectral differences in the tissue could be due to higher nucleic acid levels in the cancerous samples\(^{46}\).

Beljebbar et al\(^{47}\) (2009) used a Raman micro...
DETECTION OF CRC IN BLOOD SAMPLES

The advantage of Raman spectroscopy over other vibra-
tional techniques is its ability to analyse samples in aqueous solution with minimal background. This is advantage-
ous for the study of samples such as urine and blood as they can be analysed without changing their composition by drying.

Berger et al[55] (1999) introduced the idea that Raman had potential for the analysis of biofluids, in particular human blood. Premasiri et al[56] (2012) found that the NR spectra of whole human blood are dominated by the normal modes of either oxygenated or deoxygenated haemoglobin porphyrin macrocycle and haemoglobin. This leads to the assumption that if NR is to be used for the analysis of blood components such as malignancy specific proteins for CRC other than those associated with red blood cells (RBC) an enhancement mechanism has to be used or blood samples must have RBC removed in order to overcome this issue (Figure 3).

For example, Harris et al[57] (2009) discussed the potential of peripheral blood samples analysed by NR to provide cancer screening in head and neck cancer. Using Raman spectroscopy and LDA analysis alone the study found this technique to have approximately 65% specificity and sensitivity for the discrimination of cancer in peripheral blood samples. The use of non-enhanced Raman spectroscopy for the discrimination of blood serum between normal and CRC was first reported by Li et al[37] (2012). Li et al[37] presents a study using clinical samples from 44 colon, 46 rectum and 30 healthy controls. Raman peak parameters and fluorescence background were used along with multivariate analysis techniques such as PCR and PLSR for the dimension deduction of spectral data. Then, LDA on PC’s is used to see diagnostic performance. Three distinct, Raman peaks were found to have significance at 1029 cm\(^{-1}\), 1538 cm\(^{-1}\) and 1170 cm\(^{-1}\). The 1538 cm\(^{-1}\) peak was assigned to beta-carotene and 1170 cm\(^{-1}\) to tryptophan and phenylalanine. The average spectra from the three sample groups were then compared and the latter two peaks were shown to decrease compared to the control group. This is explained as a decrease in anti-cancer related molecules.

This study showed that it is possible to discriminate between serum samples of patients with and without CRC. The result of the PCR-LDA analysis was promising as it identified normal samples with 87.5% accuracy and 96.7% specificity and colon cancer samples with a sensitivity of 84.8%.

SERS detection methods

Raman spectra are subject to interference from samples that exhibit fluorescence; a fluorescence signal is far greater than the Raman response so it can "hide" any Raman signal. Fluorescence is fairly common when using wavelengths less than 785 nm that are commonly used in biological studies. Also, when Raman is used on complex biological samples Raman peaks can be additive, making differentiation between biological markers difficult to resolve, this results in a reliance on data manipulation in order to detect small spectral differences. SERS offers a resolution to some of these
issues as it reduces the effects of inherent fluorescence while increasing the intensity of the Raman response of the sample. Premasiri et al.\(^\text{[58]}\) (2001) demonstrated the need for an enhancement mechanism in order to detect some low concentration analytes in urine analysis by Raman spectroscopy. It was found that urea had a sufficiently high concentration to be analysed by NR in liquid form however, lower-level nitrogen compounds needed enhanced Raman spectroscopy in order to detect these compounds.\(^\text{[58]}\) Therefore SERS was used as an enhancement mechanism in order to be able to detect the compounds that were in concentrations too small to detect with Raman.

The basic principle of SERS is to amplify the Raman response of a given analyte. The SERS effect was first discovered in 1974, and understood to be an enhancement of Raman scattering in 1977\(^\text{[59,60]}\). This is generally achieved by having an analyte attached or close to the surface of a nanoscale metal substrate causing an enhancement factor of up to \(10^5\)\(^\text{[14,15,61,62]}\). The exact mechanism of SERS enhancement is still an area of active research, however it is generally accepted that two mechanisms contribute to the enhancement\(^\text{[63]}\). One is based on electromagnetic field enhancement due to excitation of electromagnetic resonances in the SERS active nanoscale metal substrate. The other is known as “chemical enhancement” which is a result of the metal electrons causing a charge transfer between the metal substrates and the adsorbates. The result of the combined enhancement is an extremely powerful technique that combines ultra sensitive detection limits with the molecular structure information from Raman spectroscopy giving the possibility of single molecule detection\(^\text{[64]}\).

**Clinical applications of SERS for CRC**

The main use of SERS in clinical applications for CRC has been as a detection method. Lin et al.\(^\text{[65]}\) (2011) were the first to report SERS serum analysis for the detection of CRC. In their study colloidal gold nanoparticles were simply mixed with serum samples from 38 patients and 45 control samples and dropped onto an aluminium substrate. This technique is known as label-free SERS. It generally relies on blood constituents being adsorbed onto the surface of metallic nanoparticles causing an enhanced Raman response. In the Lin et al.\(^\text{[65]}\) study a Raman micro-spectrometer (Renishaw, Great Britain) fitted with a 785 nm diode laser was used to gain spectra in the 300-1800 cm\(^{-1}\) range. Spectra from the two groups were normalised to the integrated area under the curve in the 350-1750 cm\(^{-1}\) wavenumber range. The mean spectrum for the normal serum and the cancer serum were then compared to isolate wavenumbers that showed the most variation between the two groups. Then an empirical diagnostic algorithm based on peak intensities at 725 cm\(^{-1}\) and 638 cm\(^{-1}\) was used to classify the normal and the cancer samples. These were chosen based on previous studies by Han et al.\(^\text{[66]}\) (2008) that showed the ratio to be important disease marker. This technique was compared with both empirical and multivariate analysis techniques. The potential for using whole spectra coupled with PCA-LDA to be used as a screening technique for CRC was then discussed using the PCA-LDA methods in a second publication from the same group\(^\text{[63]}\). The two publications also included tentative peak assignments and major vibrational bands that have been previously observed in serum samples. The peak assignments are vital to being able to accurately describe what is happening at the molecular level when a patient has a disease. However, due to the additive nature and complex compounds found in serum samples it can sometimes be difficult to be sure of peak assignments. Furthermore, the methods described above such as looking at the 725 cm\(^{-1}\) band can be a marker for disease but it is not specific to CRC. In order for the technology to become useful as a diagnostic there is a need to have a Raman/SERS marker that is specific to CRC.

The need for specific detection has motivated the development of “labelled” SERS probes. These probes have previously been used for detecting disease specific proteins in both tissue and serum samples\(^\text{[67-71]}\). They have also been used for the detection of circulating tumour cells\(^\text{[72]}\). However there is little reported on the specific application of targeted SERS probes for use in detecting CRC\(^\text{[73]}\). In general targeted SERS techniques rely on either aggregation of antibody-functionalised nanoparticles after exposure to a protein or they are used to form of sandwich immunoassay similar to that of an ELISA setup but using SERS active probes rather than fluorescent-tagged antibodies (Figure 4).

Both techniques offer advantages and disadvantages; techniques relying on the aggregation of nanoparticles use fewer antibodies and in general have very simple protocols that can be done on cheap substrates. However, when dealing with the aggregation of nanoparticles it can be difficult to “find” the correct spot on a sample where the SERS intensity is greatest. Furthermore, studies relying on aggregation can be susceptible to large variation as controlling the aggregation can be difficult. SERS based immunoassay holds the advantage over aggregation because if the disease specific protein is in a sandwich style assay then the area to probe is easier to locate and the Raman signal is less likely to be variable, as one would expect more even coverage of the protein over the assay area. One advantage of both of these techniques over current fluorescence methods is that Raman bands are much
onto the chips. Using a 632.8 nm laser excitation, measurements were taken and two peaks appeared to be prominent in the SERS spectra at 1350 cm⁻¹ and 1570 cm⁻¹. Polynomial fitting was then used to determine SERS peak height for each of the samples. In order to validate that the SERS peak height and the concentration of the serum samples were correlated the measurements were repeated at different dilutions of the same serum sample. It was found that 10-fold dilutions were the saturating dilution for the NHC chips so 10 fold dilutions were used throughout the rest of the experiment. Finally the SERS peak height for both of the prominent peaks was compared between the three groups of samples and it was found that the SERS peak heights in the benign samples was indeed significantly lower than in the cancerous samples. This was calculated using the Pearson product-moment correlation coefficient and the non-parametric Spearman’s rank correlation coefficient.

Another slightly different approach to using SERS for clinical use for CRC is using SERS as a characterisation/validation tool when developing other nanoscale devices such as the work done by da Paz et al. (2012). In this study SERS was used as a characterisation tool in the development of maghemite nanoparticles as a theragnostic device for CRC. The SERS active nanoparticles used in this study were functionalised with Anti-CEA antigen in the hope that they could be used to detect primary and metastatic CRC, it was hoped by the authors that these nanoparticles could then be developed for a variety of applications including magnetic resonance imaging (MRI) enhancement and targeted drug delivery.

**Limitations of Raman and SERS in clinical applications**

Raman and SERS based tools have shown potential that they will have a place as either an alternative or an adjunct to current diagnostic methods. The development of SERS biomarker detection could also lead to its use in personalized medicine. However, there are still some limitations of Raman and SERS techniques that will need to be overcome before they are routinely used in a clinical setting. These include: (1) Many Raman studies involve costly equipment and expensive substrates, there will need to be investigations into cost reduction for large scale applications; (2) Raman and SERS studies that are carried out in a laboratory will require sample handling and storage, the effect of handling samples and storage techniques on the performance of Raman based tools will need to be quantified; (3) Thermal damage thresholds of in vivo tissue and ex vivo tissue samples from the colon and rectum will need to be established; (4) Many studies for clinical use different analytical techniques, and still require the skill of the user to determine the results of these techniques. User-friendly software for diagnostic analysis of the spectra will need to be developed and tested for multi-user reliability; (5) Inter equipment variability studies will need to be carried out, Raman equipment can often be susceptible to variability from external factors such as room temp, laser stability, etc.; and (6) SERS based techniques have been subject
to reproducibility issues, CRC is a heterogeneous disease so if immunoassay style tools are to be used then large scale studies with clinical samples will need to be carried out.

**CONCLUSION**

In the field of cancer detection Raman spectroscopy and SERS has gone through a period of rapid progress in the last decade. The use of Raman in clinical applications for CRC has previously been dominated by the discrimination of cancerous vs non-cancerous tissue with only a few studies on the use of Raman with biofluids for CRC detection. There are currently successful in vivo Raman tools for real-time use during endoscopy. These tools can be used to gain molecular information through Raman imaging and traditional spectroscopy. Therefore, they aid current endoscopic techniques by giving extra molecular information that could potentially be missed using traditional methods. However, Raman tools are still in general expensive to produce and require specialist knowledge in order to operate the machinery. Furthermore, thermal thresholds for the damage of GI tissue need to be properly established before these tools can be ready for use in a routine clinical setting. In future, national multi-site trials that include large patient numbers are needed to study the thermal threshold of tissues.

Future research into the large-scale manufacture (and miniaturisation) of Raman tools needs to be carried out to investigate variability between sites and investigate the cost effectiveness of Raman tools compared to current technology.

In order to detect low concentration analytes SERS has started to become an alternative method to Raman. SERS offers enhanced signals and reduced fluorescence compared to Raman. Current research uses different techniques to gain a SERS response from samples. One of the limitations of SERS based techniques has been that the variations in the plasmon resonance of nanostructures that cause a SERS response are subject to large variability. Therefore, in mixing style SERS methods research into reducing the variability in SERS response even across a single sample will need to be investigated. Another method of gaining a SERS response is through a SERS based immunoassay; this has been successfully used to detect the current accepted biomarker for CRC CEA. The immunoassay design is based on reducing variability by controlling separation of the SERS substrates. SERS immunoassay has the potential to have multiplex detection of analytes in both tissue and biofluids. This could be one of the biggest areas of development if CRC research follows that of diseases such as nasopharyngeal cancer. Furthermore, if SERS is successfully used to detect different concentrations of biomarkers then this opens up the possibility into research towards personalised medicine and detecting changes in the levels of biomarkers using less invasive methods than are currently available in the United Kingdom (i.e., through blood based testing). There are currently other CRC detection tests in development that are more advanced than SERS such as mSept9 blood based testing. However, SERS based detection aims to have detection limits below the current available technology; therefore it offers the possibility of research into new biomarkers for CRC based on Raman or SERS spectral signals. Furthermore SERS and Raman based techniques still have the ability to be developed into techniques that are used in conjunction with other developing detection methods.

Both Raman and SERS techniques will also need further research into producing a universal method of background subtraction and analysis of data. Currently many research groups use different methods of data analysis that can be complex and still require clinicians to interpret results using spectral knowledge. In order for Raman and SERS based detection to be implemented into a clinical setting simple, user-friendly programs will need to be produced that remove the need for interpretation of spectra by a user. If the spectral analysis is automated then Raman and SERS techniques have the potential to become ”observer-independent” tools.

Raman and SERS techniques are currently still in development with the aim to be in regular use in a clinical setting. If the technological limitations are overcome then the techniques have the potential to produce more specific, affordable detection and screening for CRC that can be routinely used in a clinical setting as an alternative or an adjunct to current methods.

The final limitation to Raman and SERS based techniques will be that of persuading clinicians that the new technology can replace existing techniques, it is possible that national based trails showing the robustness of Raman and SERS techniques will go some way to achieving this.

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