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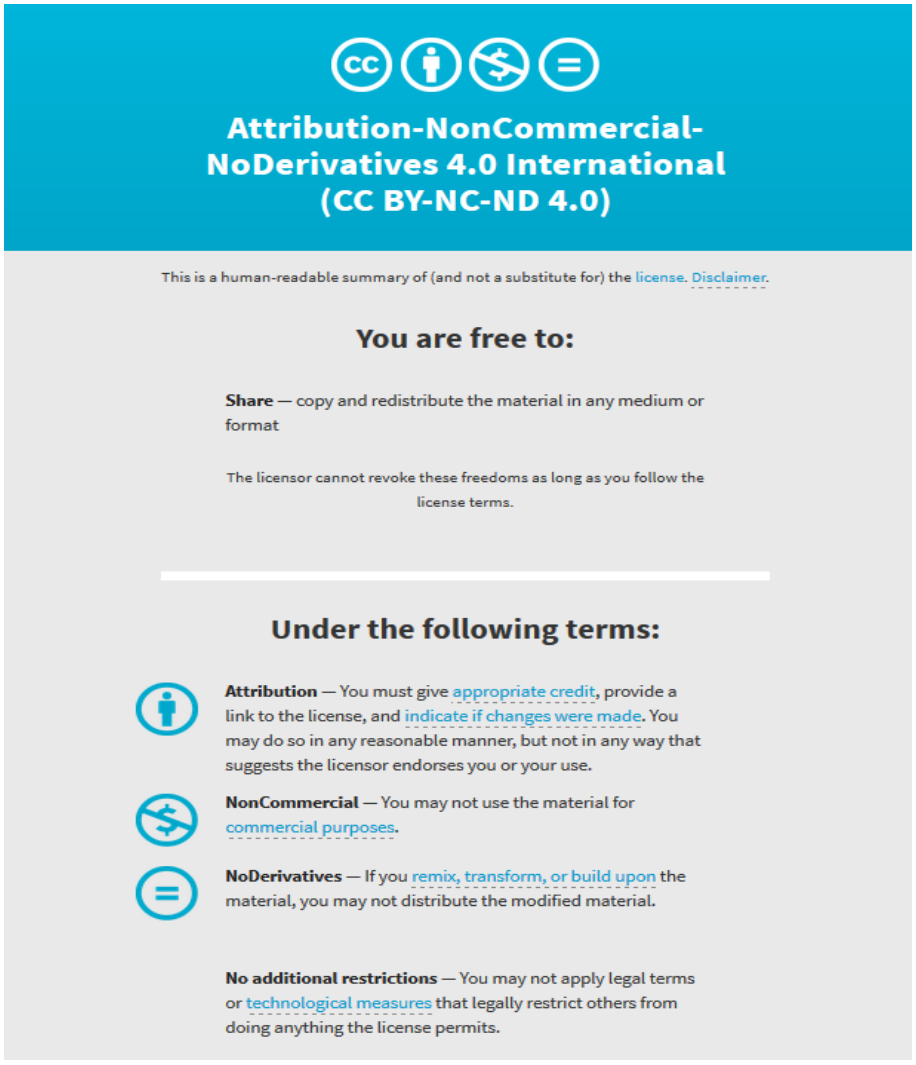

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Measuring and tracking vitamin B12: A review of current methods with a focus on optical spectroscopy

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ABSTRACT

Vitamin B12 deficiency has been associated with an increased risk of cognitive decline. This literature review explores the current methods available for measuring vitamin B12 in human blood, serum, and urine, and the need for a globally accepted reference range for vitamin B12. We present optical spectroscopy, including chemiluminescence measurements, absorption and fluorescence spectroscopy, surface plasmon resonance, and Raman spectroscopy, as a promising technique for detection and tracking of vitamin B12. Considerations for future research are highlighted, including enhancing the sensitivity of optical spectroscopy and prospective pathways to improve the reproducibility, selectivity, and speed of vitamin B12 detection.

KEYWORDS

Biophotonics; cognitive ageing; dementia; optical sensor; sensing; vitamin; vitamin B12

Introduction

Vitamin B12 (cobalamin and its derivatives, as shown in Figure 1) (1) deficiency has been associated with an increased risk of poor cognitive health (2, 3). Increased levels of vitamin B12 have been shown to reduce the likelihood of older adults transitioning from mild cognitive impairment to dementia (4) and in at least one case may help reverse the symptoms of frontotemporal dementia, as previously shown in a B12 recovery treatment program (5). The overall effects of vitamin B12 deficiency have been previously discussed in the literature (6, 7). It has been found that vitamin B12 plays an important role in 2 metabolic cycles that can affect the health of the nervous system (8). First, vitamin B12 is crucial in transferring a methyl group from 5-methyltetrahydrofolate to homocysteine (Hcy), thereby generating tetrahydrofolate (THF) – important in DNA synthesis – particularly the DNA synthesis of red blood cells and intestinal wall cells. When this process is impaired, tetrahydrofolate levels are reduced and Hcy levels are increased; increased Hcy levels can be detrimental to

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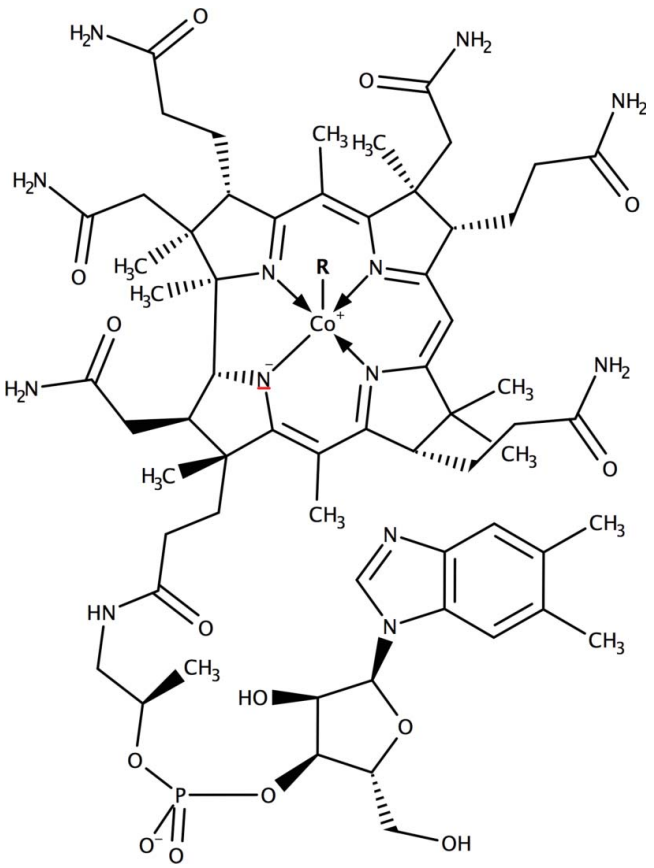

Figure 1. Chemical structure of the basic form of vitamin B12 (cobalamin). Some of its most common derivatives consist of R = –CN (cyanocobalamin), R = –OH (hydroxycobalamin) and R = –CH3 (methylcobalamin).

cognitive health (9). Secondly, vitamin B12 partakes in a reaction that converts methylmalonyl-coenzyme A (coenzyme-A linked to methylmalonic acid - MMA) to succinyl-coenzyme A (coenzyme-A linked to succinic acid), an important step in the extraction of energy from proteins and fats required for the synthesis of myelin, a material surrounding the axons of neurons that is essential for a functioning nervous system. Increased MMA levels are an indication of impaired myelin synthesis that affects the function of neurons, thereby contributing to impaired cognition (10).

The growing number of studies indicating the significance of the relationship between vitamin B12 and cognitive health cannot be ignored (11). In order to establish the mechanism that underlies this relationship, it is essential to accurately and reliably measure vitamin B12 (12, 13).

Reference ranges are essential in order to guide the interpretation of data (14), but there is no globally accepted standardized reference range or measurement technique (15). Physiological ranges of vitamin B12 in human blood serum are 200–900 pg/mL in the United States (16), where it is also noted the levels of <500 pg/mL may result in symptoms of vitamin B12 deficiency in older adults. In Australia, the reference range is 200–900 pg/mL with the limit for subclinical deficiency noted as 300 pg/mL (17). The reference range for vitamin B12 in blood serum, however, is dependent on the measurement method (18).

Governments and health organizations therefore usually focus on dietary intake guidelines for vitamin B12 (19, 20) instead of blood ranges. However, these guidelines do not account for conditions like pernicious anaemia (also known as Biermer's disease), a macrocytic anaemia that can prevent the absorption of vitamin B12 (21) and therefore result in anaemia-like symptoms, such as weakness and asthenia, even though the dietary intake of vitamin B12 is within the recommended range. Increasing age also decreases the absorption of the protein-bound form of vitamin B12 (22, 23); in Australian aged care facilities, approximately 14% of residents are reported to have undetected vitamin B12 deficiency (24). An additional tool for establishing vitamin B12 deficiency has been to measure associated biomarkers such as methylmalonic acid (MMA) and homocysteine (Hcy) for diagnostic purposes (25); but the levels of these biomarkers may not reliably indicate cobalamin deficiency when interpreted in isolation, making measurements of vitamin B12 essential for diagnosis (26).

Current methods of detecting and measuring vitamins are mostly based on microbiological and chemical techniques (27, 28) and we briefly cover those in this literature review. In recent years, there has been great progress in developing optical detection techniques that can provide rapid and precise answers across a wide range of chemical and biological target species. These detection methods include fluorescence spectroscopy (29), Raman spectroscopy (30) and surface plasmon resonance (SPR) effects (31). It is in view of these developments that we review the current status of detection techniques for vitamin B12 as they promise rapid, cost-effective and efficient detection at the physiologically-relevant range of concentrations.

Established methods

Microbiological detection

Historically, the first technique for detecting vitamin B12 has been based on microbial cultures, in which the growth of certain microorganism is monitored when exposed to different samples and compared against a calibrated growth curve for specific compounds (32). *Lactobacillus leichmannii*, microorganisms that require corrinoids as a growth factor, have been used for vitamin B12 measurements as their growth depends on externally supplied vitamin B12 (32–34). In the work by Skeggs et al. (35), which formed the basis of microbiological determination of vitamin B12 levels for decades, a culture of *Lactobacillus leichmannii* was grown for 24 h inside a liquid skim-milk-based medium with a carefully regulated pH before being added to a refined serum assay and autoclaved for 15 min at 120°C. The results, showing the growth rate of the microbes in the sample, could be read after 24-h incubation at 37°C and were compared against the growth rate curve of these microorganisms exposed to known amounts of cobalamin.

Microbiological techniques have since evolved for better precision and lower limits of detection (LOD) down to 20 pg/mL (36), although certain drawbacks for serum vitamin B12 measurements remain as additional factors like the presence of antibiotics in the blood serum affect the growth rate of the of the microorganisms and therefore the resulting estimates of vitamin B12 concentration (37).

Immunoassays

Immunoassays are biological detection techniques for the presence of specific molecules in a sample that use a specific antibody to bind the target molecule onto a substrate for further

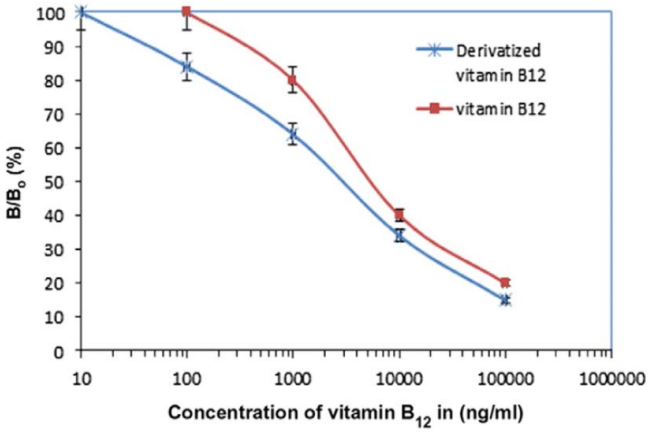

Figure 2. Standard ELISA curve for derivatized vitamin B12 versus vitamin B12. Each point represents the mean of 20 determinations. Vertical bars indicate error bars with 5% value (taken from (43)).

detection (38). The first example of an immunoassay targeting vitamin B12 in human serum appeared in 1982 (39), while further studies exist that use competitive immunoassays to measure and analyze vitamin B12 (40, 41). Direct competitive enzyme-linked immunosorbent assays (ELISAs) for vitamin B12 have been developed, where immobilized rabbit antibodies that capture cobalamin were immobilized on a cover slip and incubated for 20 h at 4°C before a biotinylated detection antibody is introduced that reacts with horseradish peroxidase-avidin and produces a color reaction that increases with increasing concentration of vitamin B12 (42), as shown in Figure 2, for example, for a standard response curve of this measurement (43). This method has a LOD of 2.2 pg/mL, although it requires lengthy preparation of the antibody plates and numerous processing steps.

Combining immunoassays with radioactive forms of vitamin B12 has also been shown to be a sensitive tool for measuring vitamin B12 by competitive radioassays (44), where vitamin B12 in a sample competes for binding onto specific antigens against a known amount of the radioactive (^{57}Co) vitamin B12 (45). While this method has been shown to be reliable (46, 47) and the LOD of 288 pg/mL is within the physiological range (48), the requirement for highly trained operators and the cost of radioisotopes and measuring equipment has limited its adoption (49).

High-performance liquid chromatography (HPLC)

HPLC is a chemical technique that separates different compounds contained within a liquid sample by passing a mixture of the sample substance along with one or more liquid solvents through a microporous column (50). Different chemical species show different retention times in passing through the HPLC column under pressure, resulting in different flow rates through the column that can be used to identify them. Vitamin B12 was first isolated using HPLC in 1997 (51) and since then there have been various studies focusing on simultaneously identifying multiple members of the B-vitamin family through HPLC (52–54). It must be noted that, due to the LOD for this technique being in the order of 80 ng/mL (52), HPLC work has primarily been used for identifying vitamin B12 in pre-made samples such as food supplements and dietary supplements (22).

Capillary electrophoresis (CE)

This is a technique in which a capillary is filled with a liquid electrolyte into which the sample under investigation has been mixed before an electric field is applied across it that separates the ions in the sample (55, 56). In the work by Lambert et al. (57), for example, high voltage (15 kV) is applied across the length of a glass capillary filled with a mixture of cobalamin derivatives capillary and electro-osmosis causes the ions in the solution to travel along the length of the capillary. The different cobalamin derivatives separate as they travel along the capillary and are detected near its end ultraviolet light (266 nm) absorption through the sample, a process that takes 25 min and results in the signatures of the compounds in the sample, as shown in Figure 3 (taken from (57)).

The signal detected was then compared against results for individual derivatives using the same measurement to calibrate the process. The LOD for detecting vitamin B12 by CE were 20 μg/mL, as described by Lambert et al. CE is often used in a complimentary fashion to HPLC to cover different concentration and complex size regimes (57).

Radioisotope and mass spectrometry

A significant body of work exists in the literature whereby a radioactive isotope of vitamin B12 is used to enable detection based on radioactivity measurements. Initial studies consist of subjects being given a radioactive isotope of vitamin B12, usually in the form of (^{57}Co) vitamin B12 (58, 59). The radioactivity of blood plasma (60, 61) or even of the whole body (62–65) is then measured and correlated to the amount of vitamin B12, with the ultimate aim of using this technique as a diagnostic tool (66, 67). More recently, a form of vitamin B12 containing ^{14}C has been used in conjunction with mass spectrometry (MS) to measure

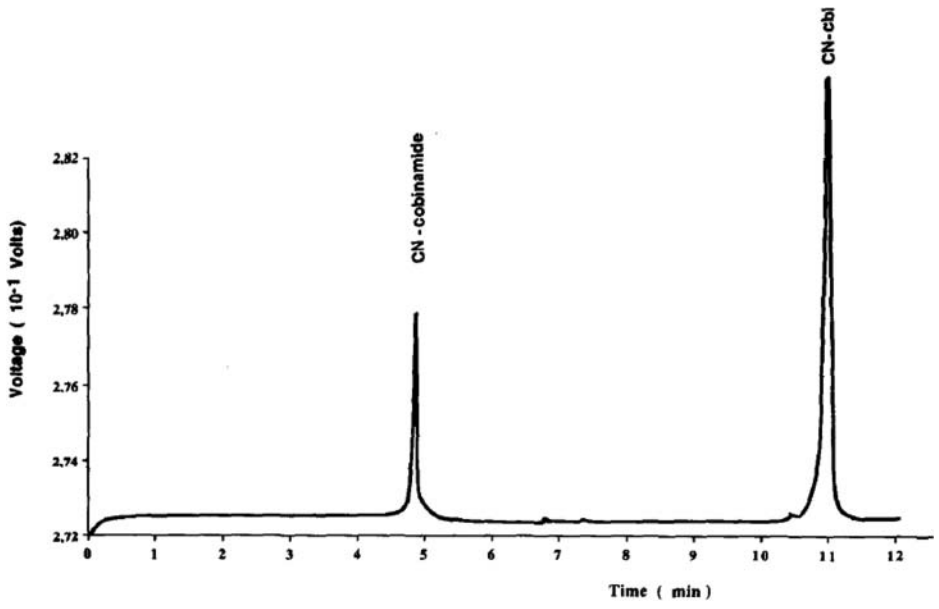

Figure 3. A signature of two different cobalamin compounds in a liquid sample as analyzed by capillary electrophoresis (taken from (57)).

human absorption of vitamin B12 (68). MS relies on ionizing a sample to create charged fragments of a chemical species that are then separated by accelerating them through an electric or magnetic field. The field deflects them based on their charge-to-mass ratio, resulting in different components arriving at different points of the detection mechanism (69). The actual chemical species are then identified by correlating the mass of detected fragments to known mass profiles of known molecules, with very low detection limits for biological samples that have been modified to include the (^{14}C) isotope (70). This technique has been used for vitamin B12 identification down to a LOD of 100 fg/mL (68), although this limit refers to detecting the modified version of cobalamin that requires a complicated and costly synthesis and a complex experimental setup, rather than the naturally-occurring form of vitamin B12.

Overall the established detection techniques have their basis in microbiology and chemistry, and some of them have indeed shown very low detection limits and selectivity. They do not, however, answer all challenges in measuring vitamin B12 that ideally requires rapid identification of vitamin B12 at a reasonable cost. It is due to these limitations that we turn our attention to a different set of detection techniques based on optical spectroscopy.

Optical detection techniques

Optical detection techniques for chemical species have established themselves as a reliable and sensitive technology for biological molecules (71) such as DNA and proteins (72) and various vitamins (73, 74). Optical detection of chemical species revolves around seeing how light interacts either directly with the target molecules or with intermediate compounds that change their behavior in the presence of the target molecules, and using this change in light properties such as the color of light they emit to identify and quantify the target species (71, 75–77). Some common optical detection techniques for vitamin detection are fluorescence detection (78, 79) and Raman scattering (80–82), where light incident on a sample changes color depending on the chemical bonds present in the sample molecules, but other optical sensing techniques have also shown great promise (83).

Chemiluminescence (CL)

Most vitamin complexes do not spontaneously emit light, with the notable exception of vitamin A (84). The same applies for vitamin B12 that is not known to emit light under optical excitation. Fluorescence detection can be deployed, however, through the interactions of vitamins with light-emitting molecules in the process of chemiluminescence. Cobalt has been shown to enhance the CL reaction between luminol and dissolved oxygen (85), or between luminol and hydrogen peroxide with a LOD of 890 pg/mL (86), as shown in Figure 4. Similar detection limits were achieved when the CL technique based on luminol was deployed on a lab-on-a-chip system, whereby flow channels are inscribed on a single substrate to enable chemical and biological measurements (87).

This CL lab-on-a-chip method promises faster readout times and a more compact geometry than conventional techniques (88). A similar technique was employed by Kamruzzaman et al. (89), who developed a microfluidic chip detector based on the reaction of luminol and silver nitrate in the presence of gold nanoparticles. This method demonstrated a LOD of 40 pg/mL.

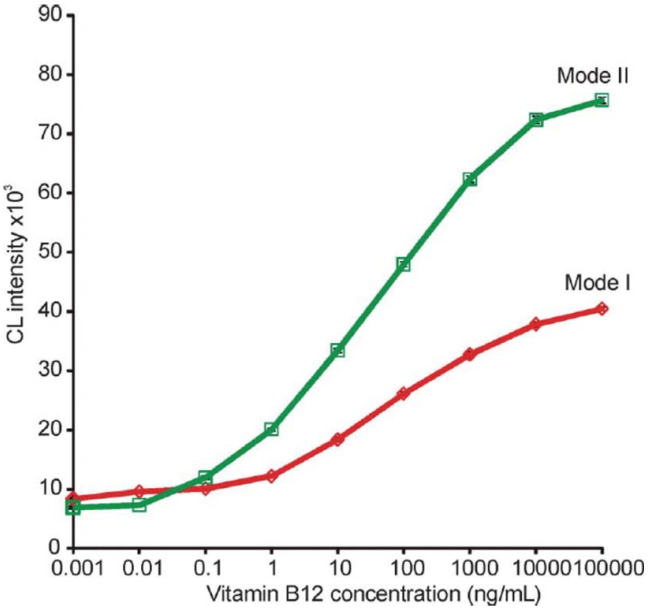

Figure 4. Concentration series for detection of vitamin B12 based on chemiluminescence in two different sensing modes (with pre-acidification of vitamin B12 taking place within and outside the microfluidic system, respectively) for a lab-on-a-chip device (taken from (88)).

Another method based on luminol was demonstrated using the cobalt (II) ion liberated from B12 as the catalyst for a luminol-percarbonate CL reaction, in this case resulting in an increase in the chemiluminescence signal with increasing B12 concentration under UV illumination (90). This technique, however, showed LOD of 9.3 ng/mL, which was improved on in subsequent work to 420 pg/mL (91).

Zhang et al. showed that vitamin B12 could also be detected using CL with dodecylbenzene sulfonate (DBS)–layered double hydroxides (LDHs) used to measure liberated cobalt (II) ions (92). This technique again showed an increasing CL signal with increasing B12 concentrations, with a LOD of 570 pg/mL. This work was of particular interest due to the increased specificity of the DBS LDHs to B12, with significantly reduced cross-sensitivity to other metal ions compared to other work on luminol-based CL detection.

Absorption and fluorescence

While vitamin B12 is a poor light emitter itself, and its intrinsic absorption at biologically relevant concentrations is too small for direct detection, its presence can affect the efficiency of other light-emitting species. Rhodamine 6G, for example, has been used to indirectly measure vitamin B12 in solution by studying the effect B12 has on the fluorescence resonance energy transfer between aridine orange (AO) and Rhodamine 6G (93), illustrated by the collected spectra shown in Figure 5 for different mixtures of AO, Rhodamine 6G and vitamin B12. Work based on this technique was able to show a LOD of 2 μg/mL (94).

Work by Shang et al. demonstrated that a fluorescent probe 4-N,N-di(2-hydroxyethyl) imino-7-nitrobenzo-2-oxa-1,3-diazole (HINBD) can be used for detection of B12, with the fluorescence quenched by B12 allowing for measurements to be performed by examining

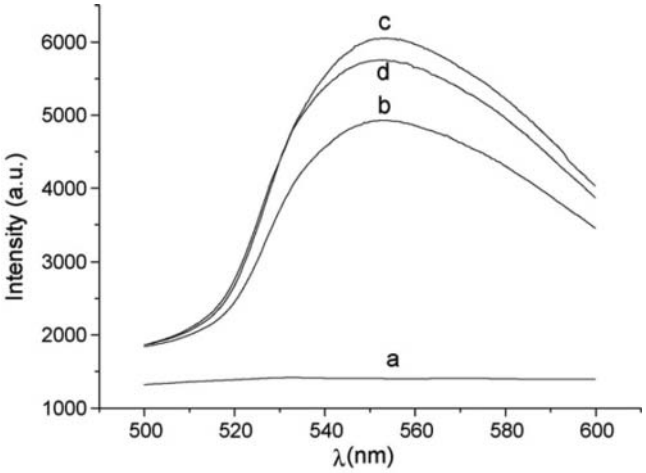

Figure 5. Fluorescence spectra using 454 nm argon laser (10 mW) as the excitation source. (a) aridine orange (AO); (b) Rhodamine 6G (R6G); (c) R6G–AO; (d) Mixture of R6G–AO and vitamin B12 (VB12) (concentrations of AO, R6G and VB12 are 1 × 10−5, 4 × 10−5 and 4 × 10−6 mol/L, respectively for (a–d)) (taken from (94)).

the intensity of the fluorophore's signal (95). This technique demonstrated a LOD of 0.1 μg/mL in water.

Vitamin B12 has also been shown to directly quench the fluorescence of CdTe quantum dots, where energy absorbed by the quantum dots is resonantly transferred to the B12 molecules (96). Carbon compounds also experience fluorescence quenching in the presence of vitamin B12 molecules, for example, when using a graphene oxide layer (97) or thermally-reduced carbon dots (98). Using these thermally reduced carbon dots Wang et al. was able to demonstrate a LOD as low as 0.1 μg/mL in an aqueous solution.

Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) sensing consists of monitoring changes in the color scattered off a metallic surface in the presence of a target molecule (99, 100). Surface plasmons are electron oscillations along the interface of a metal and a dielectric surface. The frequency of these oscillations is very sensitive to the refractive index of the environment, so in a sensor configuration the resonance frequency of surface plasmons will change depending on the refractive index of the sample medium (101). This technique usually requires an antibody that selectively binds target species on the surface of the sensor, where the resonance is strongest. SPR has been shown to be useful for determining vitamin B12 levels in various dietary supplements (102). SPR has also been used to indirectly determine the presence of vitamin B12 by monitoring the interactions between vitamin B12 and its binding proteins, with a LOD of about 1 μg/mL (103).

Raman spectroscopy

Although the optical detection methods discussed above all allow for the detection of B12 in solution, they present various issues for real-world samples or point-of-care applications.

Some techniques, luminol-based CL, for example, are susceptible to cross-sensitivity from many of the common metal ions present in biological samples. In addition, these techniques require extensive sample preparation and complex chemical reactions, greatly limiting the scope for real-world deployment.

One technique that can potentially address these shortcomings is Raman spectroscopy, an optical detection technique that directly identifies the chemical bonds that make up individual molecules in a sample (104). Raman scattering, which lends its name to the spectroscopic technique, is a phenomenon by which a small part of the light delivered onto a sample from a laser source changes its wavelength (color) by a small fraction corresponding to the vibrational energy of the chemical bonds in the sample (105). As molecules consist of multiple chemical bonds, this process results in a "Raman fingerprint," which is representative of all the bonds in a given molecule (106). By using an optical spectrometer, a device that decomposes light into its wavelength components, an identifiable signature for that particular molecule is generated (107). Most biologically relevant molecules consist of similar elements (carbon, oxygen, nitrogen etcetera); the similarity of these chemical bonds makes discriminating between them using Raman spectroscopy challenging (108). Vitamin B12 is unique in that it contains a cobalt ion linked to an organic corrin ring – a structure not found across other molecules in the human body that gives the most prominent peaks in the Raman spectrum of vitamin B12 (109, 110). This makes Raman spectroscopy a particularly attractive technique for measuring vitamin B12 because its unique Raman signature can provide direct molecule identification (111).

The first measurements of vitamin B12 using Raman spectroscopy appeared in 1973 (112, 113), where the Raman spectrum of vitamin B12 (shown in Figure 6) was identified and the technique is used to identify vitamin B12 and its derivatives in aqueous solutions (114–116). This is followed by a long hiatus until 1989, where more detailed Raman studies of vitamin B12 start emerging (117, 118) as laser sources and detectors improved.

Most studies of vitamin B12 using Raman spectroscopy have concentrated on understanding the molecular structure of the vitamin B12 molecule, for example, in identifying

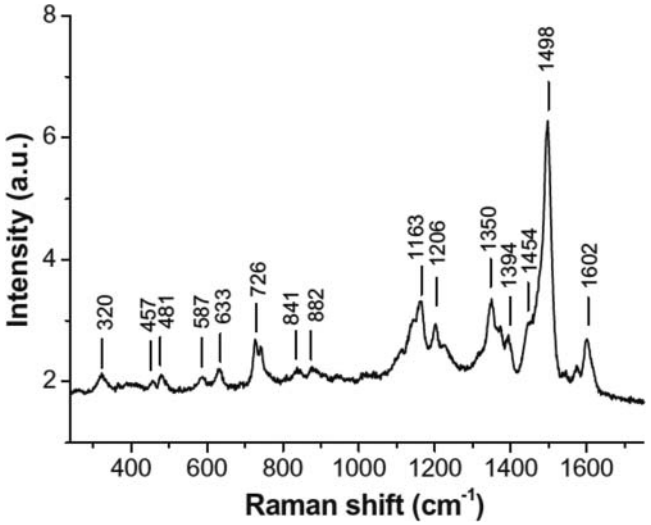

Figure 6. Raman spectrum of vitamin B12 powder (taken from (125)).

Table 1. Limit of detection (LOD) and advantages and disadvantages of optical spectroscopy techniques used for measurements of vitamin B12.

Technique	LOD (pg/mL)	Advantages	Disadvantages
Chemiluminescence	40 (89)	Fast, sensitive	Poor specificity, extensive sample purification
Fluorescence quenching	110 (95)	Fast, sensitive	Poor specificity
Surface plasmon resonance	10^3 (103)	Kinetics information	Poor specificity, extensive sample preparation, low sensitivity
Raman spectroscopy	10^6 (114)	Specificity, little sample preparation	Low sensitivity

the different vibrational modes of cobalamin (118) and its coenzymes (119). This line of research has given considerable insight into the fine details of the molecular structure of vitamin B12, for example, in characterizing and modeling of the different vibrational modes in the cobalamin molecule (111, 120, 121), and the chemical and conformational changes it undergoes in the body, for example, during its binding process to the coenzymes it helps to metabolize (122–124).

There is a notable lack of systematic studies of the LOD in Raman detection of vitamin B12 which represents a promising pathway for future research. Raman spectroscopy inherently has a low signal intensity in comparison to other optical processes such as fluorescence, with reported LOD down to 250 ng/mL (114), but enhancement techniques like surface-enhanced Raman spectroscopy (SERS) (126–128) may increase signal intensity due to interactions between cobalamin molecules and metallic substrates (125). Raman measurements of vitamin B12, therefore, have the potential to yield both useful and innovative results. A strong case can be provided by other vitamins; SERS measurements of vitamin C show a detection behavior that closely matches the performance of HPLC measurements by using a structured silver substrate to enhance the Raman signal (129), and SERS has also been identified as a reliable technique to build up libraries of spectral signature for vitamin (130). Recent approaches such as producing SERS-active biotags for the characterization of biological samples have shown that the intensity of the SERS signal can be as high as that of fluorescence – but with the added advantage of unique identification of the compound (131) which represents an exciting step towards detecting physiologically-relevant concentration of vitamin B12.

Overall these emerging optical techniques offer potential selectivity, speed and low detection limits for measurements of vitamin B12. The current detection limits for these techniques are summarized in Table 1, along with their key advantages and disadvantages.

Conclusions

Precise measurements of vitamin B12 concentration in humans to aid in diagnosis of vitamin B12 deficiency are still a field of intense research and scrutiny. Established methods are currently approaching their limits in terms of reproducibility, selectivity and speed; for these reasons determining vitamin B12 deficiency remains a time consuming and costly process. With recent advances in technology and our understanding of the interactions between light and cobalamin, some of the new optical detection techniques may develop into field-deployable devices for measuring vitamin B12 and aiding in the diagnosis of its deficiency. Rapid, reliable and reproducible measurements of vitamin B12 levels could further our

understanding of the role of vitamin B12 in conditions like dementia and Alzheimer's disease and ultimately contribute to early diagnosis and guide prophylactic actions for at-risk populations.

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