# Method for Detecting Oxidants and Reductants in A Sample

## Technical Field

The present invention generally relates to a method for detecting oxidants and reductants by observing a fluorescence color change. The method can be used in an assay format for determining free radicals and antioxidants in a sample for biomedical research or redox status research.

## Background

Free radicals and antioxidants are essential to ensure the health and well-being of a human body. Free radicals are highly reactive molecules with an unpaired electron that are constantly being produced in our bodies via biological reactions with oxygen. Studies have shown that excessive quantities of free radicals are harmful to our body and have been implicated in aging and major ailments such as cancer development, heart attacks and stroke. On the other hand, antioxidants are small molecules with reducing properties that can mitigate the harmful effects of free radicals. However, excessive antioxidants will in turn adversely affect one's health. Thus, a delicate balance of free radicals and antioxidants within the body is vital to keep one healthy. The ability to simultaneously monitor the levels of free radicals and antioxidants is therefore undoubtedly crucial for biomedical research, particularly in the areas of anti-aging and disease prevention, and many important applications in the health care, personal care, sports, as well as the food and beverage (F&B) industries, e.g., to monitor the shelf-life of products.

However, there are currently no methods based on a color-change fluorescence assay principle, that offer a

detection of both free radicals and antioxidants in a simple way to determine which of the two are present in an excess.

Most of the existing methods in the art make extensive use of specially-designed organic molecules and proteins, which have many intrinsic disadvantages. Firstly, organic molecular fluorophores are designed to target certain types of free radicals or antioxidants, making them not suitable as a general probe for diagnosing the overall balance of free radicals and antioxidants in the biological sample. In addition, their chemical synthesis procedure is often multi-stepped, tedious and requires many toxic reagents and solvents. The susceptibility of organic fluorophores to oxidation and photobleaching will give unreliable assay results after long durations of storage. Chemical stabilizers are usually added to prolong their storage stability, which can in turn interfere with the assay result. No satisfying all-encompassing solution has yet to be found for these problems that are associated with organic molecule-based assays.

Secondly, protein-based assays using intrinsically-fluorescent proteins and those where the protein is a key assay ingredient are highly susceptible to temperature and pH changes, thereby making prolonged storage difficult.

Critically, they need to be harvested from living organisms, thereby limiting their supply and increasing the cost of the assays, despite their high sensitivity and selectivity.

As a whole, all these assays cannot provide the detection capability of both free radicals and antioxidants. Therefore, separate assays have to be conducted independently when studying free radicals and antioxidants in one sample, making the process cumbersome,

tedious and time-consuming. In addition, they respond irreversibly to either oxidation (by free radicals) or reduction (with antioxidants), and hence are unable to detect processes with opposite redox chemistries occurring. Their specificity also limits detection to a particular subset of free radicals/ antioxidants which are well-studied and understood, leaving no room for presently unknown systems. Most of the existing assays are designed based on the principle of "fluorescence turn on/off" in the presence of target analytes, which is difficult to observe visually, unless a very obvious fluorescence enhancement/ quenching results.

Therefore, there is a need for easy-to-use assay systems for such detection, especially those for measurement in one-pot.

Another problem with present assays for the detection of redox properties is that they cannot be easily observed as they require complex measurements.

There is a need to detect free radicals and antioxidants in a simple way without using complex methods.

There is a further need to detect the free radicals and antioxidants in a simple way by mixing a sample with a reagent and observing the resulting physical change in the same pot.

**Summary**

According to the disclosure, there is provided a method for detecting oxidants and reductants wherein the method comprises the reaction of a sample with a substrate and observing a fluorescence color change.

According to a first aspect, there is provided a method for detecting oxidants and reductants wherein the method comprises the reaction of a sample with a metal nanocluster and observing a fluorescence color change, wherein the metal nanocluster is a mixture of metal nanoclusters templated with two different DNA sequences.

Advantageously, the method may be useful for two-way simultaneous detection of free radicals and antioxidants of a sample by a simple reversible color change

guaranteeing a comprehensive survey of the overall effects from all known and unknown free radicals and antioxidants present.

The unambiguous fluorescence color change can enhance the reliability and feasibility of visual-detection without any instrumentation.

The substrate of the method is a metal nanocluster ("mNC" or "metal NC"). The substrate serves as a surface on which other materials may be templated to. The metal nanocluster is templated with a DNA sequence.

Advantageously, this method allows for the detection of both free radicals and antioxidants simultaneously. Two opposite redox processes can be observed for their overall effect in one assay using this method.

Advantageously, this method allows the observation for a wide range of free radicals and antioxidants.

Advantageously, according to this method no toxic reagents are used and the method can be carried out in aqueous medium at room temperature. Accordingly this non-toxic method may have in-vitro and in-vivo applications.

Advantageously, this method allows for real-time monitoring of reduction and oxidation, because the color change is reversible.

Preferably, in the disclosed method the metal nanocluster may be templated with a 2 mer to 20 mer of the DNA sequence or templated with a homocytosine single-stranded DNA sequence.

Advantageously, the short DNA sequences disclosed to be usable in the method are obtainable at low cost and customizable and do not need to be harvested from any living organism, rendering the disclosed method highly versatile, cost-effective, sensitive, safe and reliable

for their application across a wide variety of both in-vivo and in-vitro sensing platforms.

The method makes use of mixtures of metal nanoclusters templated with two different DNA sequences.

Advantageously, the disclosed method allows for adapting the detection method to the specific purpose, e.g. to achieved a high sensitivity.

In another embodiment the metal of the metal nanocluster may be selected from the group consisting of silver, gold, copper, palladium, ruthenium, rhodium, osmium, iridium, platinum, mercury, rhenium, and alloys thereof, with silver being specifically mentioned.

Advantageously, these nanoclusters based substrates, especially the silver nanocluster substrates, are less susceptible to photo bleaching and oxidative quenching and are therefore stable for storage at room temperature for months.

In another embodiment of the method the color change is recorded as Emission Color Ratio (CR).

Advantageously, this method enhances the sensitivity compared to the pure visual detection by measuring quantitative CR values.

Preferably the particle size of the nanoclusters is below 2 nm. Advantageously, these small size nanoclusters show a desirable utility in the disclosed method.

According to a second aspect, there is provided an assay for detecting oxidants and reductants comprising at least one step wherein the inventive method is used.

Advantageously, the assay allows for the detection of both free radicals and antioxidants by observing a color change.

In one embodiment of the assay, the assay comprises the following two steps:

a) mixing a metal nanocluster with a sample in a medium

b) observing the fluorescence color change of the mixture.

This assay can be modified according to another embodiment of the disclosed assay to allow for the real-time observation of the mixture.

Advantageously, the assay can then be run as a simple one-pot mix-and-test format for real time observation.

According to the present disclosure, there is provided the use of the assays for detecting free radicals or antioxidants potentially relating to aging processes or diseases.

According to a third aspect, there is provided the use of the assays for detecting free radicals or antioxidants relating to aging processes or diseases.

Advantageously, the use of the assay allows for a simple way to analyze the redox properties of samples in biomedical research.

According to a fourth aspect, there is provided the use of the assays for evaluating the shelf-life of a food, beverage, health care, personal care or sports product.

Advantageously, the use of the assay allows for a simple way to analyze the redox properties such products with a use in health care, personal care, sports or the food and beverage industries.

According to the present disclosure, a corresponding assay kit for detecting oxidants and reductants in a redox system of a sample comprising at least one metal nanocluster is provided.

According to a fifth aspect, there is provided an assay kit for detecting oxidants and reductants in a redox

system of a sample according to the assays as defined above comprising a mixture of metal nanoclusters templated with two different DNA sequences.

According to the present disclosure, the use of metal nanoclusters for detecting oxidants and reductants by fluorescence color change analysis is disclosed.

Advantageously, the metal nanoclusters show surprising redox characteristics that can be used in the disclosed methods.

## Definitions

The following are some definitions that may be helpful in understanding the description of the present invention. These are intended as general definitions and should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

Unless the context requires otherwise or specifically stated to the contrary, integers, steps, or elements of the invention recited herein as singular integers, steps or elements clearly encompass both singular and plural forms of the recited integers, steps or elements.

As used herein, the term "oxidant" or "oxidizing agent" refers to an element or compound in an oxidation-reduction (redox) reaction that accepts an electron from another species. Free radicals are an example of oxidants.

As used herein, the term "reductant", "reducing agent" or "anti-oxidant" refers to element or compound that loses (or "donates") an electron to another chemical species in a redox chemical reaction.

As used herein, the term "metal nanocluster" refers to few-atom fluorescent clusters. Those nanoclusters preferably have a particle size of less than 100 nm, preferably less than 2 nm. The clusters can consist of two atoms up to the bigger sizes.

The method of the invention can make use of a new class of metal nanoclusters. The disclosed DNA sequence templated nanoclusters can be prepared according to well developed and commercially-applicable known methods such as the phosphoramidite approach and solid support synthetic protocols. Moreover, multi-well plates can be used, hence allowing highly-efficient high-throughput synthetic applications, see *Org. Biomol. Chem.*, 2005, **3**, 3851-3868 for a review.

The template DNA sequence is preferably 30 mer, or shorter, such as a 2 to 20 mer. The DNA can be single-stranded or double-stranded with single-stranded DNA being preferred. The DNA sequence is preferably cytosine rich. A homocytosine DNA sequence is most preferred.

The metal nanoclusters show fluorescence. The fluorescence is preferably in the visible wavelength area. A color change means a shift in the fluorescence wavelength. For example, due to an oxidation reaction caused by atmospheric oxygen, a color change phenomenon termed as "fluorescence blue-shift" may occur. The opposite effect, termed as "fluorescence red-shift", may occur due to a reduction reaction.

The metal nanoclusters can be used as mixtures of nanoclusters from different DNA sequence templates (e.g. mixture of C10-silver nanoclusters with C20-silver nanoclusters). The color of the nanocluster mixture can quantified by an emission color ratio (CR) calculated based on the quotient between the sum of green and blue fluorescence intensities relative to the red fluorescence intensity.

The nanoclusters can be used to react in a medium with a sample to form an assay. Such medium can be an aqueous medium, such as water. The reaction can take place at different temperatures, with room temperature (about 25 °C) being preferred.

The metal of the metal nanocluster may be preferably a noble metal. More preferably, the metal may be selected from the group consisting of silver, gold, copper, palladium, ruthenium, rhodium, osmium, iridium, platinum, mercury, rhenium, and alloys thereof. Silver is most preferred in the disclosed method. In addition, the metal nanocluster can consist of more than one type of metal.

Unless specified otherwise, the terms "comprising" and "comprise", and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, unrecited elements.

As used herein, the term "about", in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically

disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Certain embodiments may also be described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the disclosure. This includes the generic description of the embodiments with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

**Detailed Disclosure of Embodiments**

Exemplary, non-limiting embodiments of the disclosed conductive fibrous material will now be disclosed.

According to a first aspect, there is provided a method to detect oxidants and reductants wherein the method comprises the reaction of a sample with a substrate and observing a fluorescence color change.

In one embodiment, the method relates to the simultaneous detection of oxidants und reductants. This embodiment is used, if the sample contains both oxidants and reductants.

In an embodiment, the oxidant may be selected from the group consisting, but not limited to, free radicals, hydrogen peroxide, oxygen, and other naturally-occurring oxidising substances produced by the body.

In an embodiment, the reductant may be selected from the group consisting of sodium borohydride, ascorbic acid (Vitamin C), aspartic acid, oxalic acid, formic acid, carbon monoxide and other naturally-occurring chemicals

with chemically reducing properties such as catechols and polyphenols.

In an embodiment, the substrate may be selected from the group consisting of a metal nanocluster, silica and polymer, which serves as a surface on which other materials may be templated to. In a further embodiment, the substrate may be at least one metal nanocluster templated with a DNA sequence. The metal may be preferably a noble metal. The noble metal may be selected from the group consisting of silver, gold, copper, palladium, ruthenium, rhodium, osmium, iridium, platinum, mercury, rhenium, and alloys thereof. In the most preferred embodiment a silver nanocluster is used. The silver nanoclusters advantageously show a strong fluorescence color, excellent photo stability and high emission rates. Silver is preferential due to its unique chemical ability to bind strongly to DNA bases such as cytosine in its reduced form as elemental silver atoms or its precursor as silver cations. Furthermore, the nanoclusters produced have the right photophysical properties that allow it to fluoresce strongly in the visible electromagnetic spectrum.

The size of the particles may be in the micron-range or in the nano-range. Where the size of the particle is in the micron-range, the particle is termed as a microparticle and where the size of the particle is in the nano-range, the particle is termed as a nanoparticle.

The size of the nanoclusters may be selected from the list consisting of about 0.1 nm, about 0.2 nm, about 0.3 nm, about 0.4 nm, about 0.5 nm, about 0.6 nm, about 0.7 nm, about 0.8 nm, about 0.9 nm, about 1.0 nm, about 1.2 nm, about 1.4 nm, about 1.6 nm, about 1.8 nm and about 2.0 nm. The nanoclusters may consist of about less than 500 atoms, about less than 400 atoms, about less than 300

atoms, about less than 250 atoms, about less than 200 atoms, or about less than 150 atoms.

In one embodiment, the template DNA sequence may be selected from the group consisting of about 2 mer to about 250 mer, about 2 mer to about 20 mer, about 10 mer to about 250 mer, about 10 mer to about 200 mer, about 10 mer to about 150 mer, about 10 mer to about 100 mer and about 20 mer to about 250 mer. The DNA sequence may be single-stranded, double-stranded or a mixture thereof. In a further embodiment, where the DNA sequence is single-stranded, the DNA sequence may be selected from the group consisting of homocytosine single-stranded DNA sequence, homoadenine single-stranded DNA sequence, homothymine single-stranded DNA sequence, homoguanine single-stranded DNA sequence and mixtures thereof in any permutations and lengths.

In one embodiment, the color change is recorded as Emission Color Ratio (CR) indicating a shift from red to green and blue or from green and blue to red.

In this embodiment the color ratio is calculated as: CR = [Fluorescence intensity of green light (wavelength 540nm) + Fluorescence intensity of blue light(wavelength 490nm)]/ Fluorescence intensity of red light. (Formula 1)

In one embodiment, a mixture of metal nanoclusters templated with two different DNA sequences is used in the disclosed method. The mixing ratios can be varied over a broad range as needed for a specific detection. Typical ratios are selected from the list consisting of 0.1:1, 0.5:1, 1:1, 3:5, 5:3 and preferably 0.5:1 to 5:1 and most preferred 3:5 to 5:3. As an example a mixture of an AgCN templated with a 10 mer DNA sequence and an Ag CN templated with a 20 mer DNA sequence can be mentioned. Preferred mixing ratios are then 10:1 to 1:10 and more

preferred 8:2 to 2:8. Most preferred is a mixture wherein the ratio is about 4:6.

Further a set of embodiments to be mentioned is:

A method to detect oxidants and reductants wherein the method comprises:

(a) metal nanoclusters (mNCs) templated with DNA sequence

(b) a fluorescence color change arising from mNCs undergoing a redox process (either oxidation or reduction)

(c) a one-pot mix-and-observe assay format where the mNCs are simply mixed with the sample of interest.

The method according above wherein the DNA is of any sequence that can give mNCs a two-way redox-responsive fluorescence color change. The method where the color change is recorded as Emission Color Ratio (CR value), which can be used to monitor the presence of oxidants and reductants. The assay can consist of one type of mNCs templated by a specific DNA molecule (e.g. just C20), or of a mixture of two types of mNCs (e.g. 4:6 mixture in Example 3) template by different DNA sequences. The method wherein the metal used is any known cluster-forming metal, including but not exhaustive to silver, gold, copper and palladium. The method where the mNC can consist of just one metal, or a mixture of multiple nanocluster-forming metal. The method where the sample of oxidants and reductants can consist of free radicals and antioxidants respectively. The method where the sample can be pure (containing only one compound) or a mixture (containing 2 or more compounds). The method where the mixture can consist of all molecules having the same redox property, or of molecules having different redox chemistries.

In another embodiment, the method is used in an assay for detecting oxidants and reductants simultaneously comprising the following two steps

a) mixing a metal nanocluster ("mNC") with a sample in a medium

b) observing the fluorescence color change of the mixture.

In one embodiment, the medium may be selected from the group consisting of an aqueous medium, an organic solvent medium or a mixture thereof. Where the medium is an aqueous medium, the medium may be selected from the group consisting of a biological sample such as urine and blood plasma. In order to be mixed with the medium a preferred embodiment uses the metal nanocluster in an aqueous solution. Advantageously the mNC may be stored in such medium for long periods of time selected from the group consisting of about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks and about 7 weeks.

In one aspect, the observation may take place in the same medium container where the mNC is mixed with the sample ("one-pot process"). In another aspect, real-time changes of the redox system can be observed in the one-pot process.

In another embodiment, the disclosed assay may be used to detect the existence of free radicals in a sample. Advantageously, the disclosed method allows for such detection in the presence of antioxidants, even if their nature and amount is unknown. The disclosed method and assays are useful for in-vivo detection.

In one embodiment, the assay may be used to determine the relative reduction strength of an agent, for instance an inorganic or organic oxidizing or reducing agent.

According to another aspect, a corresponding assay kit for detecting oxidants and reductants in a redox system of a sample comprising at least one metal nanocluster is provided. In the embodiments according to this aspect the same metal nanoclusters can be used as described for the above methods and assays. The assay kit may comprise of container containing the metal nanocluster together with other parts, such as for instance a packaging, instructions on how to run the assay, vials for mixing with a sample etc.

According to another aspect, the use of metal nanoclusters for detecting oxidants and reductants by fluorescence color change analysis is disclosed. In the embodiments according to this aspect the same metal nanoclusters can be used as described for the above methods and assays.

The disclosed methods and the assay based thereon may be useful in biomedical research and other applications where redox systems and the content of free radicals are of interest for observation, such as the food and beverage industry, the personal and health care industries, etc. Probes for new sensing applications can be based on the disclosed method as well as new imaging applications.

## Brief Description Of Drawings

The accompanying drawings illustrate a disclosed embodiment and serve to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

Fig. 1a shows the fluorescence spectrum of C20-templated AgNCs before (dashed lines) and 1 hour after

(solid lines) sodium borohydride ($NaBH_4$) addition, showing an increase in red fluorescence intensity accompanied by a decrease in green and blue fluorescence.

Fig. 1b shows a comparison between the reduction capability of reducing agents by the decrease in CR values (left to right: $NaBH_4$ > ascorbic acid > aspartic acid) and their corresponding color changes (i.e., cyan to red, yellow-green and green, respectively) in fluorescence.

Fig. 2a shows a schematic illustration of the color-change assay principle of using red fluorescent C10-AgNCs for detecting tryptophan radical under UV irradiation. Photographs and CR values (inserts in Fig 2a and b) calculated from the corresponding emission spectrum of C10-AgNCs in the presence of tryptophan (a) with and (b) without UV light irradiation, confirm that the red-to-cyan color conversion of C10-AgNCs is due to the presence of free radicals but not the atmospheric oxygen.

Fig. 3 shows a palette of fluorescence colors in the mixture of C20- and C10-templated AgNCs (left) and their respective color change in the presence of free radical (middle) and antioxidant (right).

Fig. 4 shows a schematic diagram (a) illustrating assay principle for two-way color change detection of (b) free radicals (i.e., hydroxyl radical), giving a positive gradient in the CR-sample concentration standard plot; and (c) antioxidants (i.e., Vitamin C), giving a negative gradient in the standard plot, obtained 30 minutes after addition of analyte to the 4:6 AgNC mixtures.

Fig. 5a shows a schematic diagram showing the assay principle for diagnosing the overall redox properties of free radical-antioxidant sample using yellow-fluorescent 4:6 AgNC, which turns cyan with excess free radicals and red with excess antioxidants. Fig. 5b shows a plot of change in CR value (CRanalyte - CRAgNC), where a positive

value indicates excess free radicals and a negative value being diagnostic of excess antioxidants present. Values are obtained after 30 minutes of incubating the sample with the 4:6 AgNC mixtures.

Fig. 6 shows a real-time monitoring of free radical generation from the 0.2 mM tryptophan sample under UV irradiation for (a) 5 minutes and (b) 1 minute as well as (c) a control sample without UV irradiation. Vitamin C was added at 15 minutes of the monitoring process to study the free radical scavenging of the antioxidant, indicated by decreasing CR values in all samples (a-c) with time. A 4:6 AgNC mixture was used as the fluorescent color change probe for the assay.

**Examples**

Non-limiting examples of the invention and a comparative example will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the invention.

**Preparation Example: Synthesis and color inter-conversion properties of homocytosine templated silver nanoclusters (AgNCs)**

Synthesis of fluorescent AgNCs involved the incubation of homocytosine single-stranded DNA with silver nitrate ($AgNO_3$) for 20 minutes, followed by the addition of sodium borohydride ($NaBH_4$) to form Ag atoms that interact with the DNA template to form AgNCs with well-defined fluorescence colors. The fluorescence arises from the discrete molecular energy levels that arise from metals in the dimensions of approximately 1-2 nm. All reagents are used as received from Sigma Aldrich. Surprisingly, for the

20-mer homocytosine (C20), a color conversion of red-fluorescent AgNCs to stable cyan-fluorescent AgNCs (a mixture of green and blue-emitting species) during the course of reaction (within 24 hrs) due to an oxidation reaction caused by the atmospheric oxygen was observed. This color change phenomenon is referred to as "fluorescence blue-shift" in the following. Reversibly, these cyan fluorescence AgNCs can be converted back to red by chemical reduction (termed as fluorescence "red-shift"), the opposite process to oxidation. Free radicals, by virtue of their unpaired electrons, are known oxidizing agents while antioxidants neutralize free radicals by their reducing (electron donating) properties. The unique redox-responsive color inter-conversion property of homocytosine templated AgNCs was used to develop novel reducing agent and oxidizing free radical detection assays as shown in Example 1 and 2, respectively. Using different lengths of oligocytosine DNA as template, the final emission color of stable NCs can be tuned synthetically. For example, C10 template renders the formation of red fluorescent AgNCs, which can be used in combination with the stable cyan fluorescent C20-AgNCs for simultaneous detection of free radicals and antioxidants by tuning their initial fluorescence color in a mixture, as illustrated in Example 3. The color of the AgNCs mixture can be quantified by an emission color ratio (CR) calculated based on the quotient between the sum of green and blue fluorescence intensities relative to the red fluorescence intensity at any instant. By this definition, an increase in CR value indicates the presence of oxidizing agents while a decreased value indicates the presence of reducing agents. It has been empirically determined that a CR value of above 1.8 gives a distinct cyan fluorescence, while that below 0.2 exhibit red

fluorescent. When the CR value falls within the range of 0.2 to 1.8, a whole host of intermediate colors, namely orange, yellow and green, can be observed. Most importantly, this CR value will be useful to unambiguously quantify any color changes in the sample fluorescence that is hard to be differentiated by the naked eye.

Based on the CR value and visible fluorescence color changes, it has been demonstrated in the following examples that the application of the novel fluorescence color-change assays to diagnose the overall redox property of a free radical-antioxidant mixture (Example 4), and to monitor the ongoing free radical generation process and radical-scavenging effects of antioxidants (Example 5) is possible.

**Example 1: Detecting reducing agents using cyan-fluorescent C20-templated AgNCs**

This is an example for the application of cyan-fluorescent C20-templated AgNCs for detecting reducing agents, as indicated by an increase of red emission intensity at 640 nm wavelength accompanied by the decreased of both green and blue fluorescence intensities at ($\lambda$em = 540 nm) and ($\lambda$em = 490 nm) respectively, as shown in Figure 1a. The relative reduction strength of reducing agents can be determined by the extent of fluorescence red-shift of the AgNCs sample, via both a visible color change (when viewed under a UV lamp) as well as by a decrease in the CR value. As shown in Figure 1b, the addition of sodium borohydride, a strong reducing agent, to the C20-AgNC samples caused an obvious fluorescent color from cyan (CR = 2.0) to red (CR = 0.15) in 1 hour. The next strongest reducing agent, ascorbic acid, renders a moderate extent of fluorescence reddening, with an

observable color change from cyan (CR = 2.0) to yellow-green (CR = 1.2). The weakest reductant is aspartic acid with the least obvious change in color and CR value (from 2.0 to 1.8). The results unambiguously show the relative reduction strengths (redox potentials) of sodium borohydride ($NaBH_4$) > ascorbic acid > aspartic acid, which is in agreement with the literature reports determined by other methods (Int. J. Electrochem, 2012, Article ID 276246; J. Phys. Chem. B, 2006, 110, 15700-15707). It is noteworthy to mention that even for the extremely weak reducing agent like aspartic acid, the change in CR value is still detectable and sufficient to indicate the redox nature of the sample, thereby showing the sensitivity of this assay.

**Example 2: Detecting free radicals using red-fluorescent C10-templated AgNCs**

This is an example of another type of stable red fluorescent AgNCs using C10 as template for free radical detection that do not spontaneously convert to cyan under atmospheric oxygen. A tryptophan radical, which is formed from the amino acid tryptophan under UV irradiation is used. As shown in Fig. 2a, when the tryptophan solution is irradiated with UV light, the highly oxidizing tryptophan radicals are formed, which are able to give an obvious blue-shift in fluorescence color of the C10-templated AgNCs sample in 5 minutes, indicated by an increase of CR value from 0.03 to 1.5. In the absence of UV irradiation, however, the CR values of the sample remain the same at 0.03, as seen from the unchanged red fluorescent color of the sample (Fig. 2b). Monitoring the control sample for up to an hour, no noticeable fluorescent color changes from red are observed demonstrating the ability of our current

assay in detecting free radicals under atmospheric oxygen conditions.

**Example 3: One-pot, two way detection of free radicals and antioxidants**

In the previous two examples, it has been demonstrated that C20-(possible reduction to red) or C10-(possible oxidation to cyan/blue) templated AgNCs can be used to detect samples with known reducing or oxidizing properties through their respective fluorescence color change. To detect a sample with unknown redox properties, it is essential to produce an AgNC sample with a stable intermediate color between red and cyan, e.g. yellow, to give a visually detectable red-shift (yellow-to-red) or blue-shift (yellow-to-cyan) of fluorescence color that is not achievable using C20-AgNCs (cyan) or C10-AgNCs (red) alone. A mixed-NCs assay that is capable of providing distinctive emission color changes for the detection of reductants and oxidants at the same time in one-pot is shown in this example. Table 1 shows the mixed AgNCs samples consisting of stable cyan-fluorescent C20-AgNCs and red-fluorescent C10-AgNCs in different proportions (i.e., 10:0, 8:2, 6:4, 4:6, 2:8, and 0:10 of C20:C10 AgNCs). This simple fluorescence color tuning method does not require additional reagents or changing the AgNC synthesis conditions which could affect the redox properties of the NCs and their detection capabilities. Furthermore, the redox properties of an unknown sample can be easily and unambiguously determined by the gradient of CR-vs-analyte concentration plot (see example 4b) and increase the reliability of current assay from visual detection.

**a) Detection of free radicals/ antioxidants via simple visual observation using mixed NCs**

Table 1 shows the fluorescent color detection of hydroxyl radicals (generated from UV irradiation of hydrogen peroxide) and antioxidant (i.e., vitamin C) using the mixed NCs of different ratios. Opposite directions of color conversion was observed in the presence of vitamin C (towards red) and hydroxyl radicals (towards cyan). Of which, the most obvious two-way fluorescence color change occurs with the sample containing a C20:C10 AgNC ratio (by volume) of 4:6 (boxed in Fig. 3). This particular NC-mixture ratio gives an initial stable yellow fluorescence color which shows a distinct color change signature in the presence of free radicals (blue-shift) and antioxidants (red-shift). In subsequent examples, this optimal yellow fluorescent C10-C20 AgNC mixture is abbreviated as the "4:6 AgNC mixture".

**b) Detection of free radicals/ antioxidants via gradient of CR value-versus-analyte concentration standard plot**

When the 4:6 AgNC mixture (CR =0.6) was mixed with different concentrations of free radicals or antioxidants, distinct signs of the slope/ gradient of the CR value-sample concentration standard plot were obtained (Fig. 4). In the presence of hydroxyl radicals, the gradient of the plot is positive, as a higher concentration of hydroxyl radicals oxidizes more red-emitting AgNCs to green and blue-emitters, giving a larger blue-shift of the fluorescent color in the mixture. However in the presence of antioxidants, the gradient of the plot obtained is negative. From this example, it is found that other than a distinct visually-observable color-change signature obtainable, the gradient of the CR value-versus-analyte concentration plot obtained can be diagnostic of the overall redox property of a system as well. The distinct

diagnostic signatures obtained as demonstrated with the label-free fluorescence color change assay in this example allow the assay to simultaneously detect free radicals and antioxidants via a fluorescence color change (non-FRET based method. With this mixed NCs assay, the state of redox stress of an unknown system could be unambiguously diagnosed at a glance without the cumbersome task of having to use separate free radical and antioxidant assays for a similar purpose.

**Example 4: Diagnosing the overall redox properties of free radical-antioxidant mixtures**

In biological samples, a mixture of free radicals and antioxidants are usually present. However as noted previously, a person's health and well-being rests on a very delicate balance between these species present, easily upset by the diet and the supplements consumed. Hence, it is pertinent that the overall antioxidant and free radical activity of a biological system can be assessed as a whole, rather than subjected to independent assays for free radicals and antioxidants in order for effective and reliable data to be obtained for biomedical and health sciences applications. The NCs assay disclosed herein is capable of this assessment to indicate if antioxidants or free radicals are in overall excess, both situations ultimately being detrimental to a person's health and well-being (Fig. 5). To illustrate this sensing principle, hydrogen peroxide (a free radical precursor) and an antioxidant (Vitamin C) were mixed in various ratios for testing using the yellow fluorescent 4:6 AgNC mixture. Under 5 minutes UV light irradiation, some hydrogen peroxide is converted to hydroxyl radicals, which

can be sequestrated and neutralized by the vitamin C antioxidant if it is available. In Fig. 5, the difference in CR value before and after the sample addition ($CR_{analyte}$ - $CR_{AgNCs}$) is plotted against the composition of the analyte mixture. The samples containing 100% and 80% $H_2O_2$ have more hydroxyl radicals than can be captured by the Vitamin C available, resulting in them being overall oxidative in nature, as seen from the positive values of CR value differences obtained. In an actual biological sample (obtained from a human urine sample, for instance), it was indicated that the system is susceptible to excess cellular oxidative damage, increasing one's risk of diseases to cancer. The other four samples have an excess of vitamin C even after all the hydroxyl radicals present have been consumed, resulting in them being overall chemically reductive. Biologically speaking, this puts the person at risk of ailments associated with excessive antioxidants, such as an increased risk of stroke. This example illustrates the convenience of the disclosed NC assay when applied an unknown mixture containing both free radicals and antioxidants, eliminating the need for separate radical and antioxidant assays routinely employed for such analyses in the biomedical and health sciences industries.

**Example 5: Real-time monitoring of free radical generation and capture**

In Examples 1 to 4, the wide-ranging applications of redox-responsive AgNCs for in-vitro sensing of free radical and antioxidant simultaneously at a fixed time was demonstrated.

However, in many biological studies, real-time monitoring of free radical generation and capture during a dynamic biological processes is essential. For instance, to study

the increasing oxidative damage by free radical accumulation in skin cells generated by continuous UV exposure, one will need to test the effects of different durations of UV irradiation on the oxidative stress levels of skin epithelial cells, as well as the effects of an antioxidant-containing product for reducing the extent of UV-induced damage. In this example, it is demonstrated that the disclosed fluorescent NCs assay is applicable of real-time monitoring of the effects of continuous UV irradiation on a very dilute aqueous solution of tryptophan -a small molecule commonly found in cellular proteins which has been implicated in cancer development under UV irradiation due to the formation of highly oxidizing radical (Environ. Mol. Mutagen., 2011, 52, 81-104), and the effects of free radical scavenging of the antioxidants by subsequently adding vitamin C as shown in Fig. 6.

From Fig. 6, the ongoing oxidative damage from continuous UV irradiation can be easily detected by a continuous increase in CR value. A long duration (5 mins) of UV irradiation leads to a greater extent of oxidative damage (CR value increase from 0.6 to 5.5) than a short (1 min) UV exposure (CR value increases from 0.6 to 1.6). In contrast, no oxidative damage was observed for the system not subjected to UV irradiation (constant CR value). During UV irradiation, the tryptophan radicals continuously produced causes an unceasing conversion of red-emitting AgNCs to the green and blue-emitting species, giving rise to the fluorescence blue shift observed. Biologically speaking, this prolonged UV-induced oxidative stress results in extensive free radical damage, potentially increasing a person's susceptibility to cancer. Subsequently when the vitamin C antioxidant was added to all 3 samples, a decrease in the CR value is

observed for all samples, with the largest decrease observed for the sample subjected to the longest UV irradiation. Other than chemically scavenging the free radicals present, Vitamin C actually reverses some of the oxidative damaged caused by the free radicals generated as seen from the conversion of some of the green and blue-emitting AgNCs (oxidative damage products) back to the red-emitting species, hence giving a drop in CR value. This example demonstrates the versatility of our as-developed assay- not only can it be used to monitor an ongoing process in real time, it can also shed light on any additional processes which may occur during the process (in this present case, the addition of vitamin C reverse the effects of UV-induced damage), potentially leading to new discoveries. It is important to also note that this is the first assay disclosed to be able to detect drastically different processes (of different chemistries) with simply a one-time application of the assay material (AgNCs).

**Applications**

The disclosed method can be used in various biomedical assays or assays for investigating the presence of oxidants for stability evaluation purposes in the food and beverage industry, sports industry or personal care industry.

The disclosed method can make use of metal nanoclusters. The metal nanoclusters may be templated with a DNA sequence may be made by a simple and straightforward preparation process which can be conducted at low cost on a large-scale. Assay kits including them can potentially be commercialized as the clusters have a shelf life of several months.

It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

## Claims

1. A method for detecting oxidants and reductants wherein the method comprises the reaction of a sample with a metal nanocluster and observing a fluorescence color change, wherein the metal nanocluster is a mixture of metal nanoclusters templated with two different DNA sequences.

2. The method according to claim 1, wherein the DNA sequences are a 2 to 20 mer.

3. The method according to claims 1 or 2, wherein the DNA sequences are selected from the group consisting of homocytosine single-stranded DNA sequence, homoadenine single-stranded DNA sequence, homothymine single-stranded DNA sequence and homoguanine single-stranded DNA sequence or mixtures thereof.

4. The method according to claim 1, wherein the metal of the metal nanocluster is selected from the group consisting of silver, gold, copper, palladium,

ruthenium, rhodium, osmium, iridium, platinum, mercury, rhenium, and alloys thereof.

5. The method according to claim 4, wherein the metal of the metal nanocluster is silver.

6. The method according to any of claims 1 to 5 wherein the color change is recorded as Emission Color Ratio.

7. The method according to any of claims 1 to 6 wherein the oxidant is a free radical and the reductant is an antioxidant.

8. The method according to any of claims 1 to 5 wherein the nanocluster has a particle size of 0.01 nm to 10 nm, 0.01 nm to 8 nm, 0.01 nm to 6 nm, 0.01 nm to 4 nm, 0.01 nm to 2 nm, 0.1 nm to 10 nm, 1 nm to 10 nm, 0.1 nm to 8 nm, 0.1 nm to 6 nm, 0.1 nm to 4 nm and 0.1 nm to 2 nm.

9. An assay comprising at least one step wherein a method according to any of claim 1 to 8 is used.

10. An assay according to claim 9 comprising the following two steps

a) mixing a metal nanocluster with a sample in a medium

b) observing the fluorescence color change of the mixture.

11. The assay according to claim 10 wherein the observation of the mixture is performed in real-time.

12. Use of an assay according to any of claims 9 to 11 for detecting free radicals or antioxidants relating to aging processes or diseases.

13. Use of an assay according to any of claims 9 to 11 for evaluating the shelf-life of a food, beverage, health care, personal care or sports product.

14. An assay kit for detecting oxidants and reductants in a redox system of a sample according to the assays of any claims 9 to 11 comprising a mixture of metal nanoclusters templated with two different DNA sequences.