Detecting UV-induced nucleic-acid damage

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HIGHLIGHTS

- Many methods measure and detect DNA damage
- Fluorescent techniques are some of the newest methods applied to DNA damage
- Fluorescent hybridization probes are easy to use and low cost
- Fluorescent probes have the highest sensitivity and specificity to DNA damage

ABSTRACT

Nucleic acids (NAs) play a vital role in numerous biological processes. Absorption of ultraviolet (UV) light by NAs can result in DNA damage at the molecular level, leading to mutagenesis, carcinogenesis, and cell death. Thus, much effort has been focused on developing methods for detecting damage in NA. This article discusses the different methods used for detecting UV-induced NA damage and compares them regarding their selectivity and sensitivity to the damage induced in NAs and their advantages and limitations.

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1. Introduction

The sun emits energy at wavelengths that range through 16 orders of magnitude (10^{-4}–10^{12} nm) [1]. The vast majority of this energy is biologically irrelevant; short-wavelength radiation, such as high-energy particles, X-rays, and gamma rays are expended by atomic collisions in the upper atmosphere, and long-wavelength radiation, such as far infrared, microwaves, and radio waves, do not have sufficient energy to influence biochemical reactions. Although UV radiation comprises a minute portion of the total solar output of energy, its biological impact is immense.

Nucleic and amino acids are considered the primary chromophores of UV radiation. RNA and proteins are encoded by DNA and are readily replaced by the cellular machinery. However, DNA requires an intact, long-lived copy of itself for replication, and any errors made during the replication process can result in mutation, loss of fitness, and cell death. Such errors would result in acute and chronic health effects. Tanning and sunburn are well-known examples of the acute effects of excessive UV exposure. Skin aging and cancer represent the chronic outcomes of excessive exposure. The importance of UV damage and its repair in humans is exemplified by genetic diseases that greatly increase the risk of sunlight-induced skin cancer. In one such disease, xeroderma pigmentosum [2], failure in the DNA-repair process is associated with a major increase in the rate of squamous and basal cell carcinoma and melanoma.

UV radiation is composed of three regions [1,3]. UVC (200–280 nm) is not present in ambient sunlight, as wavelengths below ~295 nm are absorbed by ozone and oxygen molecules in the stratosphere [4]. However, UVC light has been of major importance in experimental studies of DNA photochemistry. It is readily produced by low-pressure mercury sterilizing lamps. The peak wavelength of mercury sterilizing excitation (254 nm) coincides with the peak of DNA absorption (260 nm), so it causes maximum photochemical damage to DNA.

Almost 10% of UVB (280–320 nm) radiation reaches the Earth's surface. It overlaps the high-wavelength edge of DNA and protein absorption, and is the UV range mainly responsible for direct environmental and pathological photochemical damage to DNA through the formation of DNA photoproducts. It is therefore a potent, ubiquitous carcinogen responsible for much of the skin cancers in the human population today [5,6].

UVA (320–400 nm) is photocarcinogenic and is involved in photoaging but is weakly absorbed directly by DNA and proteins. In contrast to the direct induction of DNA damage by UVC and UVB light, UVA produces damage indirectly through highly reactive chemical intermediates [7]. UVA induces the formation of singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\cdot-}$) radicals, the latter being the precursor of hydrogen peroxide (H_2O_2) involved in the generation of hydroxyl radical ($^{\bullet}OH$) via Fenton type reactions [8,9]. These intermediates react in turn with DNA to form monomeric photooxidation products, such as 8-oxo-7,8-dihydroguanine, and strand breaks and DNA-protein cross-links [4].

The relationship between the frequencies of the photoproducts and their biological effects depends on the cytotoxic and mutagenic potentials of the individual damage sites. Hence, even though a photoproduct may occur at a low frequency, its structure and location may elicit a potent biological effect. It may not alter the genetic code and, hence, not affect normal metabolism, or it may produce a truncated or partial RNA transcript encoding a dysfunctional protein. Also, it may result in activation of an oncogene or inactivation of a tumor-suppressor gene, thereby initiating the carcinogenic process [10].

Examples of DNA photoproducts that are directly induced by UV light are cyclobutane-pyrimidine dimers (CPDs), pyrimidine (6–4), pyrimidone photoproducts (6–4PPs) and Dewar valence isomers [11]. In addition to the photodimers of purine bases, monomeric photoproducts, such as the pyrimidine photohydrates, are also formed. The mechanism of their formation upon DNA irradiation with UV light has been previously studied [12,13].

In this article, we focus on reviewing different methods used for the detection of the DNA photoproducts produced due to direct exposure to UV light.

1.1. Detection of UV-induced DNA damage

Numerous methods have been developed for specific and general detection and quantification of DNA photoproducts. Methods for detecting individual lesions (Fig. 1) require quantitative acidic hydrolysis (base release) or enzymatic digestion (release of nucleosides, nucleotides, or short oligonucleotides), followed by separation of the individual lesions of interest from the overwhelming majority of normal DNA constituents. Finally, the complex mixture of DNA lesions is analyzed either by gas chromatography-mass spectrometry (GC-MS) [14], or by coupling high-performance liquid chromatography (HPLC) to MS [15,16] or electrochemical detection methods [17,18]. Such methods have been previously reviewed in detail by Cadet et al. [12,13,19,20], so we mention such methods only when comparing them with the rest of the methods described later in the text.

In this review, we focus mainly on the general methods in which the DNA is kept intact and the lesions are measured by hybridization probes, immunological methods or the nicking activity of DNA-repair enzymes in association with sedimentation and gel-sequencing techniques that quantify the number of strand breaks.

1.1.1. Polymerase chain reaction (PCR)

PCR is one of the most reliable techniques for detecting DNA damage, as amplification stops at the site of the damage [21]. Ligation-mediated PCR (LMPCR) allows analysis of DNA at the nucleotide level of dimeric pyrimidine photoproducts and particularly of CPDs [22–24]. In subsequent steps, terminal transferase-dependent PCR (TDPCR) has been used for the mapping of 6-4PPs [25,26]. TDPCR depends on cohesive-end ligation to the 3′ ends of DNA molecules resulting from primer extension, followed by controlled ribonucleotide tailing by terminal deoxynucleotidyl transferase [25]. TDPCR has the advantage of using low doses of UVC for mapping 6-4PPs over LMPCR. An immuno-coupled PCR (ICPCR) assay has been used by Karakoula et al. [27] to estimate T<>T (cyclobutane thymine dimer) formation at the gene level and to compare gene and global levels of T<>T within human genomic DNA. A PCR-based short interspersed DNA element (SINE)-mediated detection method was developed by Wang et al. [28] for UVB-induced DNA damage and repair detection in mammalian genomes, which utilize the abundance, the dispersion and the conservation of SINEs. This assay is also based on the template activity of the DNA region between SINEs, which is amplified by using primers bound to the SINE [28].

PCR methods are very reproducible and, due to the amplification factor, can reach sensitivities for detecting double-stranded or single-stranded damage of femtomoles (fmol) or less [21–28]. Complications with PCR detection of DNA damage include potential repair of some lesions, introduction of other lesions from uncontrolled mutagens during the many replication cycles, and the need to use many primers if the sequence is not determined (i.e., for damage “mining”).

1.1.2. Immunological detection

Immunological detection has long been recognized as a potentially powerful tool in the analysis of genotoxin-modified DNA. As early as 1966, Levine and co-workers prepared antibodies against UV-induced photoproducts of DNA [29]. Since then, antibodies have been derived against several DNA damage sites, including CPDs and 6-4PPs [7,30]. The main advantages of immunochemical detection are specificity (especially if monoclonal antibodies are used), sensitivity (sub-fmol level), and simplicity once antibodies have been generated. Like other haptens, modified bases must be linked to macromolecules to elicit an immune response.

The principal methods employed in the preparation of immunogens are the synthesis of modified nucleosides or nucleotides that are then covalently coupled to proteins, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin, and the modification of single- or double-stranded DNA, which is then electrostatically bound to methylated BSA.

Problems of cross-reactivity have occurred when damaged polymeric DNA has been used as an immunogen. The antibody's specificity is not sufficient to assess individually the relative frequency of each of the four possible photoproducts for each of the three classes of bipyrimidine lesions, providing only semi-quantitative results. This became evident when a polyclonal antiserum raised against UV-irradiated DNA recognized all the thymine- and cytosine-containing CPDs together with 6-4PPs, but with a different affinity for each major class of damage [31]. Thus, if damaged DNA is to be used as the immunogen, it is important to choose conditions that greatly favor the production of a single type of damage over any other. For example, the ratio of CPDs to other photoproducts can be enhanced by acetophenone photosensitization of DNA when the DNA is exposed to 313-nm radiation via the triplet reaction channel [7,32].

Numerous monoclonal and polyclonal antibodies have been raised against CPDs, 6-4PPs and related valence Dewar isomers for ELISA, radioimmunoassay (RIA) and immuno-dot-blot measurements in nuclear DNA together with immunostaining detection of photoproducts in tissues [33–37]. The most commonly used technique is ELISA, in which the primary antibodies, when bound to the antigen, are detected by secondary antibodies linked covalently to an enzyme, such as alkaline phosphatase or peroxidase. Substrates for the enzymes are added and yield products with intense absorption at visible wavelengths. Other substrates have used primary antibodies coupled to radioactive isotopes in radioimmunoassays (RIAs) or secondary antibodies conjugated to fluorescent compounds, such as fluorescein isothiocyanate (FITC).

Immunodetection has been particularly effective for studying the induction and repair of UV-induced photoproducts. Nikaido and co-workers [35,38] established monoclonal antibodies recognizing CPDs and 6-4PPs. Using autoradiography to detect tritiated antibodies, they were able to observe 6-4PPs in human cells exposed to very low radiation doses. In contrast to normal proficient human cells, which removed more than 80% of their initial damage within 4 h post irradiation, cells derived from a patient with xeroderma pigmentosum showed almost no repair within 8 h [35]. In the detection of the very low quantity of CPDs in bacterioplankton and marine viruses caused by UVB radiation, RIA was found to be very effective [39–41]. Specific RIAs were used to monitor antibody-binding sites associated with CPDs and 6-4PPs. Using this technique, the biological role of 6-4PPs can be measured [42]. CPDs were detected in active *Mycobacterium parafortuitum* and *Serratiamarcescens* cells, using fluorescent Alexa Fluor 488 and radiolabeled ^{125}I secondary antibodies as reporters [4].

With an ELISA protocol [35], T<>T are shown to be excised from DNA in irradiated human cells more slowly than 6-4PPs; removal of the latter was virtually completed within 12 h of post-irradiation, whereas, at 24 h, half the T<>T still remained. DNA damage can be detected and quantified very efficiently by an immuno-slot-blot system utilizing chemiluminescent detection [43], secondary antibodies conjugated to alkaline phosphatase enzymes [44] and secondary antibodies conjugated to radioactive iodine [45]. Antibodies to modified nucleosides are also possible [46]. The immuno-slot-blot assay is used to detect very low levels of adduct in very small amounts of DNA. It is a very sensitive and specific assay. Recently, it was used to detect CPDs, 6-4PPs damage sites and their Dewar valence isomers in UV-irradiated mammalian cells [47].

Immuno techniques have high sensitivity and are easy to use, but generation of the antibodies can be a laborious and difficult process. As mentioned above, specificity can be a significant issue with antibodies and immune-detection techniques. Immuno techniques for detecting DNA damage can be used on both single-stranded and double-stranded DNA, although melting is often necessary for the latter to expose the damage site. Sensitivities are typically sub-fmol and can almost reach single-molecule levels. Specificity is typically limited to nucleobase-localized damage, including abasic sites, but no antibodies have yet been developed for strand breaks.

1.1.3. Gel electrophoresis

Electrophoretic techniques can approach fmol sensitivities or even lower when coupled with other damage-detection methods, such as immuno techniques (see above). However, electrophoretic techniques depend on changes in mobility and are most sensitive to strand breaks, which dramatically change DNA mobility, particularly single-stranded DNA. Thus, purely electrophoretic techniques are much less sensitive to base-localized damage and could be considered complementary to immune-detection techniques.

Many types of photodamage can be converted into single-strand breaks in DNA by enzymatic or biochemical treatment. Strand breaks induced directly in DNA by UV and breaks produced at sites of photoproducts by targeted enzymatic or biochemical procedures can be quantified by agarose gel electrophoresis. The sensitivity of these procedures requires maintenance of high-molecular-weight DNA throughout the extraction and analytical procedures. Specific cleavage at the lesion site can be achieved by using purified glycosylase endonucleases from various prokaryotic hosts, primarily *E. coli.* These enzymes combine a glycosylase that cuts the base from the sugar, leaving an apyrimidinic (AP) site, and an AP endonuclease that cleaves the phosphodiester backbone on either side of this site. These enzymes range from the CPD-specific T4 phage endonuclease V [48,49] and 8-oxo-7.8-dihydroguanine-specific oxoguanine DNA-glycosylase to more broad-spectrum reagents, such as endonuclease III and formamido glycosylase, which cut a variety of photohydrates and photooxidatively generated products [50]. Non-enzymatic cleavage of alkali-labile sites, such as AP sites or Dewar pyrimidinones, also produces quantifiable strand breaks using alkaline agarose gel electrophoresis. Pulsed-field gel electrophoresis was used most recently as the physical technique of choice for detecting strand breaks, but it suffers from low sensitivity and irreproducibility from laboratory to laboratory [51].

1.1.4. Comet assay

The comet assay is a single-cell gel-electrophoresis technique based on the principle that nicked DNA releases fragments that migrate farther in an electric field than undamaged DNA [52]. Cells are embedded in agarose, layered on glass slides, and treated with a cell-lysis solution to liberate DNA. Slides are then exposed to alkali to denature DNA and electrophoresed under alkaline conditions. Following electrophoresis, gels are neutralized and stained with cyber green, ethidium bromide, propidium iodide, or other suitable fluorescent dye to visualize tails of DNA extending from cells [53]. Images are captured using a fluorescence microscope equipped with an imaging system. These tails have the appearance of comets that increase in length and size as DNA damage increases. Tailing may be quantified by measuring tail length, tail area, or tail moment, which is the product of the comet length and the fraction of fluorescence in the tail [54,55].

Lesion-specific endonucleases extend the usefulness of the method to investigate different kinds of damage. For example, T4 endonuclease V for UV-induced CPDs was used and the comet tail length and area were significantly increased, and were easily discernable from background after subjecting a keratinocyte cell line to UVB [56]. Also, in a modified comet assay, CPDs were detected with an indirect immunofluorescence detection using a specific monoclonal antibody [57]. DNA repair can be studied by treating cells with damaging agent and monitoring the damage remaining at intervals during incubation [56]. An important feature of the assay is that damage is detected at the level of individual cells. Also, combining the comet assay with fluorescent *in situ* hybridization (FISH), using labeled probes to particular DNA sequences, increased the resolution of the assay and allowed detection of DNA damage and repair at the level of single genes or DNA sequences [58,59].

Comet assays are almost always performed on double-stranded DNA and are typically sensitive only to strand breaks. Combining comet assays with nucleobase-localized damage-specific enzymes can increase the range of damage detection by comet assays, but provides no specificity as to particular types of damage at each damage site. Sensitivity is not typically discussed with comet assays, although there is a clear correlation between comet-tail length and amount of damage. Compared to the methods discussed in this review, the comet assay is the least sensitive.

1.1.5. Absorbance techniques

DNA has a main absorbance band around ~260 nm, which represents the π–π* transitions of nucleobases. The 260-nm absorbance band is seen to decrease with UV irradiation time. Monitoring the absorbance changes of the 260-nm absorbance band of a DNA sample exposed to UV irradiation and correlating these spectral changes with UV-exposure time can be used to measure the kinetics of UV-induced DNA damage [60,61]. These results indicate that UVC-induced damage to the oligonucleotides is due to the formation of CPDs and 6-4PPs, resulting in a loss of the C_5=C_6 double bond [60,61]. In addition, an absorbance band at ~330 nm indicates the formation of the 6-4PP and is seen to increase with increasing irradiation time [60,61]. Comparing spectral changes of unirradiated controls with UV irradiated and mismatched samples shows significant spectral differences. This allows the detection of UV-induced DNA damage [60,61] and DNA mismatches.

A colorimetric method [62] for the detection of DNA damage was developed by using hemin-graphene nanosheets (H-GNs), which were synthesized by adsorbing hemin on graphene through π–π interactions, so they possess both the ability of the graphene to differentiate between damaged and undamaged DNA and the catalytic action of hemin. The extent of coagulation of H-GNs differs in the presence of damaged DNA from that with undamaged DNA due to the different amounts of negative charge exposed on their surfaces. This affects the solubility of H-GNs. As a result, the corresponding centrifugal supernatant of H-GN solution showed a different color in the presence of 3,3′,5,5′-tetramethylbenzidine and H_2O_2. This technique is used to detect the amount of damage caused by styrene oxide, $NaAsO_2$ and UV radiation on DNA. Styrene oxide was found to cause the most damage to DNA [62].

Another absorbance method was recently developed for the detection of UV-induced DNA damage through using the hypochromism probe (Fig. 2) [63]. The method depends on the hypochromic effect [64,65], which is the decrease in UV absorbance of DNA associated with the better stacking of purine and pyrimidine nucleobases in double-stranded oligonucleotides compared to single-stranded ones. The method uses a DNA hairpin probe (Fig. 2), which is

complementary to the DNA target of interest. The probe forms a hybrid with the undamaged target and the absorption signal is significantly decreased. Because of the increased instability of the hybrid when damaged, the probe will preferentially dehybridize from the target and acquire the hairpin structure, increasing the concentration of single-stranded damaged target and increasing the absorption signal. Thus, the more damaged targets in the solution, the lower the number of double-stranded hybrids and the higher the absorption signal. The hypochromism probe proves to have superior selectivity to detect UV-induced oligonucleotide damage over conventional molecular beacons and is inexpensive, making it an attractive tool for high-throughput qualitative analysis for UV-induced DNA damage. However, this method is not very sensitive.

In general, these characteristics of absorbance techniques promoted their use in analytical assays for DNA damage detection, but forced a move to other, more sensitive techniques, such as fluorescence measurements. Fluorescence techniques preserve the advantage of simplicity, but the cost of the technique will depend on the cost of the fluorescent probe used.

Absorbance techniques generally have the lowest sensitivity to damage of all the techniques discussed here, with the exception of the comet assay. Absorbance techniques can quantify amounts of damage, subject to their limited sensitivity and dynamic range, but need to be used in conjunction with other methods to yield types and locations of damage. We know of no report of such a use of absorbance techniques for DNA damage, except for the crude use of absorbance to distinguish 6-4PPs from other UV-induced photoadduct products, such as the CPDs and photohydrates.

1.1.6. Fluorescence techniques

The high sensitivity of fluorescence detection, due to the zero background nature of the technique, allowed the development of numerous bioanalytical fluorescent assays for measuring UV-induced DNA damage. Fluorescence assays for probing DNA damage have been proposed. Typically, fluorescence methods offer enhanced sensitivity and the potential for use *in situ* or *in vivo* [21]. We discuss below some of the recently developed fluorescent assays for the detection of UV-induced DNA damage.

1.1.6.1. Affinity capillary electrophoresis with laser-induced fluorescence detection (CE-LIF).

Immunochemical recognition with capillary electrophoresis (CE) and laser-induced fluorescence detection (LIF) was used to detect thymine glycols in irradiated A 549 cells (a human lung-carcinoma cell line) with a reported limit of detection (LOD) as low as 10^{-21} mol [66]. The fast (seconds), efficient separation and a small sample volume (nL range) requirement of CE with the sensitive detection of LIF combined with the specificity provided by monoclonal antibodies to a single lesion limits sample manipulation to DNA extraction, incubation with antibodies, and CE. This approach can therefore reduce potential artifacts caused by chemical or enzymatic DNA digestion, and it makes affinity CE-LIF a potentially powerful technique for bioanalysis, perhaps with single-molecule detection [67]. However, the specificity of the antibody to thymine glycols has never been definitively demonstrated.

Affinity CE-LIF was successfully used for the detection of CPD damage in human normal fibroblast CRL-2522 cells after UVB irradiation at low doses relevant to environmental exposure [68]. In addition, CRL-2522 cells were exposed to two different DNA-damaging agents, UV light and benzo(a)pyrene. Comparison of the result between non-combined and combined exposures did not show significant differences between formation of benzo(a)pyrene adducts and

UV-induced damage sites in cells. Unfortunately, the determination of the 6-4PPs sites was impossible because of the high background from the cellular DNA [68].

1.1.6.2. Fluorescence intercalators and DNA binders. Cosa et al. [69–71] showed that PicoGreen, a DNA-binding dye, can be used to detect DNA damage via its intrinsic fluorescence lifetime and lifetime sensitivity to its environment. Ionizing radiation damage to several DNA samples led to their unwinding via the introduction of single-strand and double-strand breaks, expelling the PicoGreen from the high-fluorescence bound DNA-dye complex to the low-fluorescence solvent environment. They were able to measure DNA damage from doses of 5–100 Gy with a dye:DNA base ratio of 1:10.

1.1.6.3. Fluorescent hairpin probes

1.1.6.3.1. Molecular beacon (MB) probes

MBs are oligonucleotide probes with a stem-loop structure (Fig. 2), containing a fluorescent dye on one end and a quencher on the other [72–75]. In the absence of target DNA, the fluorophore and the quencher are in close proximity, so there is minimal fluorescence due to Förster resonance energy transfer (FRET) from the fluorophore (donor) to the quencher (acceptor). However, in the presence of the complementary target sequence, the MB hybridizes with the target, resulting in a significant increase in fluorescence. If the target is subjected to UV light, UV-induced DNA damage sites will be formed, and the hybrid formed between the MB and the damaged target is less stable, since the DNA target now contains damage sites, making it less stable in its binding to the MB loop. Thus, the fluorescence is lower for this hybrid compared to that formed between the undamaged DNA and the MB. In 2005, Yarasi et al. [60] demonstrated the use of DNA MBs in the detection of UV-induced damage in poly dT and poly rU strands. The fluorescence intensity of the damaged DNA decreased with the increase of UV-exposure time, hence increased damage while the fluorescence of the unirradiated DNA controls was constant [60].

These inherent properties make MBs highly sensitive and very selective probes for DNA damage. However, MBs suffer from some limitations [76–81]. First, they require site-specific labeling of both termini of the hairpin with a fluorophore and a quencher, which makes their synthesis difficult and expensive [76,77,79,81]. Second, with its two ends occupied, any further modification would require the incorporation of an additional modified nucleotide into the stem [76,79]. In addition, due to incomplete synthesis, some of the hairpins may be only labeled with the fluorophore. Highly-sensitive assays are affected by a high background due to unquenched probe molecules [76,77]. Third, in the presence of a mixture of undamaged and damaged DNA, the MB hybridizes only with the undamaged target. Thus, the fluorescence intensity decreases with increasing amounts of damage, providing a signal inversely proportional to the amount of damage (i.e., negative detection of DNA damage). Fourthly, DNA MBs are subjected to endogenous nuclease degradation, non-specific binding by DNA/RNA-binding proteins and stem disruption [80]. We discuss attempts to overcome such limitations below.

SPs (Fig. 2) are hairpin oligonucleotide-probe molecules just like MBs and function similarly. But, unlike MBs, SPs have 2'-deoxyguanosine nucleotides replacing the quencher attached to the other end and the fluorescence quenching of the fluorophores occurs via photoinduced intramolecular electron transfer [78–81]. However, in the presence of the complementary target sequence, the stem unwinds, forcing the fluorophore and the 2'-deoxyguanosine nucleotides far apart, thereby restoring fluorescence.

Oladepo et al. [61] demonstrated that SPs have the ability to detect UV-induced DNA damage and single-base mismatches. Indeed, SPs are less costly than MBs and allow the introduction of further modification on the free terminus of the SP. However, SPs suffer from high fluorescence background, in addition to the other MB limitations mentioned above.

LNA MBs are DNA MBs that contain different numbers of LNAs (Fig. 2) in the hairpin that is complementary to the DNA target of interest [82]. LNAs are NAs containing ribonucleosides in which a methylene group connects the $C_{4'}$ and $O_{2'}$ atoms of the ribose sugar, locking it into the 3' endo (North) conformation [83]. Such probes appear to be more selective towards base-pair mismatches [83] and have been used to enhance the specificity of probes for FISH assays, DNA microarrays, and real-time PCR [84–87]. The factors affecting the selectivity of LNA MBs to detect UV-induced NA damage were optimized. Results show that high ionic strength and low target concentration improve the performance of MBs in detecting UV-induced DNA damage. Increasing the LNA ratio in the MB design leads to a decrease in the selectivity of these MBs to detect damage. This study [82] shows that, although LNA MBs have greater selectivity to single base-pair mismatches than conventional MBs, they have lower specificity for detecting UV-induced NA damage.

The chMB (Fig. 2) is another type of backbone modification that was recently studied to detect UV-induced DNA damage [88]. In the chMB, RNA bases replaced DNA bases of the conventional MBs in loop regions complementary to the oligonucleotide target. Typically, the thermal stability of RNA duplexes and RNA-DNA hybrid duplexes is greater than that of DNA duplexes when their sequences are identical [89]. Because of this, the chMB-DNA hybrid is expected to have a higher fluorescence signal than typical MB-DNA hybrids. The DNA stem is kept in the chMB to provide a reasonable melting temperature that allows the stem to open in the presence of the undamaged oligonucleotide target. This study [88] shows that the RNA bases incorporated in the MB loop increased the stability of the hybrids formed between the chMB and the oligonucleotide targets compared to that of the conventional DNA MB, allowing the chMB to have sensitivity and selectivity superior to the DNA MB. However, the chMB (Fig. 2) still suffers from some important limitations of MBs, such as the need for site-specific labeling of both termini of the hairpin and the negative detection of DNA damage (Fig. 3). For this reason, probes that do not require terminal labeling and can positively detect DNA damage (i.e., fluorescence intensity increases with the increase of damage) have been developed in order to address these specific limitations.

1.1.6.3.2. Hairpin probes

2-Aminopurine (2AP) hairpin probes [90] (Fig. 2) were also recently designed for the detection of DNA damage, so that 2APs are incorporated in the loop of the hairpin probe with the two hairpin termini free. 2AP is a fluorescent analogue of adenine which shows no fluorescence for undamaged DNA and fluorescence for damaged DNA. The probe forms a hybrid with the undamaged target and the fluorescence is significantly quenched. While the hybrid with the damaged targets is unstable, the 2AP hairpins will preferentially acquire the hairpin structure, emitting maximum fluorescence. Thus, the more damaged DNA targets are present in solution, the more 2AP probes will be in the hairpin structure and the higher the fluorescence intensity will be. This study shows that the 2AP hairpin probe is a sensitive tool to detect UV-induced oligonucleotide damage. The more 2AP nucleotides incorporated in the probe, the more the sensitivity and the selectivity of the 2AP probes appear to be enhanced, allowing these probes to have selectivity superior to conventional MBs. In contrast to MBs, 2AP

probes have overcome most of the MB limitations, mainly the feasibility of further modification at any of the two termini of the hairpin and positive detection (Fig. 3) of DNA damage.

The use of terbium (III) fluorescence in developing a sensitive, selective and inexpensive probe for UV-induced damage in NAs was recently introduced [91]. With the aid of a DNA hairpin probe complementary to the oligonucleotide of interest, the Tb^{3+}/hairpin probe (Fig. 2) is applied to detect UV-induced DNA damage. The hairpin probe hybridizes only with undamaged DNA, while the damaged DNA remains single-stranded. The single-stranded DNA can bind and enhance the intrinsic luminescence of Tb^{3+}, producing a detectable signal directly proportional to the amount of DNA damage (Fig. 2). This study shows that the Tb^{3+}/hairpin probe has superior selectivity and sensitivity to DNA damage than conventional MBs. In addition, the Tb^{3+}/hairpin probe is almost an order of magnitude less expensive than MBs, and it allows the positive detection of UV-induced DNA damage and the possibility of modifying the hairpin further. These advantages allow the Tb^{3+}/hairpin probe to overcome the main limitations of MBs and introduce this probe for the detection and the quantification of various forms of DNA damage in multiplexed assays.

Fluorescence-based techniques for detecting DNA damage show the highest potential sensitivity of all techniques, reaching the single-molecule level, but have so far been limited to fmol sensitivity. Only when fluorescence techniques are coupled with other methods, such as affinity chromatography or antibodies, can they be used for identifying the type of damage that has occurred. However, their ease of use and low cost are attractive advantages for their use in many assays of DNA damage.

2. Comparison of different techniques

The sensitivity of different techniques discussed above toward the damage DNA sites is affected by several factors. Table 1 presents a comparison between different techniques used for the detection of DNA damage sites. For example, techniques that include several sample-pretreatment steps prior to analysis, as in chromatographic and MS detection, would have high sensitivity to DNA-base damage. But such steps would increase the complexity of the technique, making it elaborate and time consuming. In addition, the greater the number of sample-pretreatment steps, the more artifacts may be introduced.

Table 1 also shows other techniques that are limited to detection of strand breaks, such as gel electrophoresis, comet assay and electrochemical detection. In these techniques, specific reagents are used to induce strand breaks at the site of damage. Such reagents may as well affect the undamaged bases, thus, introducing positive error to the detection. Also, Table 1 shows some techniques that include a separation step prior to detection, as in CE-LIF and [^{32}P]-post-labeling, while, for other techniques, such as ELISA and fluorescent DNA probes, the sample is directly detected without any sample-pretreatment or separation steps. Indeed, including a separation step prior to detection increases the sensitivity of detecting DNA-damage sites, as shown in Table 1, but it also adds to the complexity and the cost of the technique.

Techniques such as ELISA and fluorescent DNA probes have the advantage of the intrinsic bimolecular affinity of the antibody-antigen interaction or DNA hybridization. This imparts the technique its high selectivity of detection. In addition to being simple mix-and-read assays that can be performed in most laboratories with minimum requirements of instrumentation with the cost of the technique mainly depending on the cost of synthesis of the antibodies or DNA probes. It is also worth mentioning that the LODs recorded in Table 1 for different techniques

are in moles, i.e., techniques that allow measurements of small volumes of samples (nL range), as in CE-LIF, would have a lower LOD and, thus, higher sensitivity than techniques that require the use of larger sample volumes (μL range), as in fluorescent hybridization probes.

3. Conclusions and future aspects

This brief review illustrates that much effort has been focused on developing methods for detecting UV-induced damaged NAs. However, most of the proposed methods consist of multi-step procedures, are limited to specific types of damage, require expensive instruments, and/or suffer from a high level of interference. Ideally, a single damage-detection technique would provide all of the information required, including the site of damage, the amount of damage, and the type of damage. However, such an ideal technique is still to be developed.

For double-stranded DNA damage, immuno techniques coupled with electrophoretic separation offer the highest sensitivity and specificity for the lowest cost and ease of use. HPLC-MS is still the technique of choice, as it can provide type, location and amount of damage, although the cost and the difficulty of the technique are high. For single-stranded DNA damage, hybridization probes offer a low-cost, easy method for detecting the amount of damage, subject to saturation effects in the assay. Immuno-electrophoretic techniques can provide amounts and types of damage at a higher sensitivity, but with a concomitant increase in difficulty, cost and possibility of introducing more lesions in the assay process.

Ultimately, the best technique may be to use fluorescence hybridization probes as a shotgun approach to determine damage amounts in an easy way, and then follow up with immuno-electrophoretic or HPLC-MS techniques to obtain greater insight into the types and the locations of damage. However, fluorescence hybridization is still limited to the detection of damage in single-stranded DNA. The use of peptide-NA [92] openers may allow for this technique to be expanded to double-stranded DNA in the future.

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Captions

Figure 1. General scheme for the quantification of individual DNA lesions. Bullet points indicate methodological options at each stage. IS, MS, TLC, and EC are abbreviations for internal standard, mass spectrometry, thin layer chromatography and electochemical detection, respectively

Figure 2. Schematic diagram of the stem-loop structure of the molecular beacons and the hairpin probes.

Figure 3. Schematic diagram of the positive and negative detection of UV-induced DNA damage. “F” and “A” denote fluorescence and absorbance, respectively.

Table 1. Comparison of Sensitivities of Various Techniques Used for Detection of UV-induced DNA damage

Technique	Sensitivity*	Advantages	Limitations	References
ELISA	0.9 fmol	• Simple • High selectivity to specific UV-induced damage sites	• Cross-reactivity of antibodies to other types of damage or undamaged bases • Expensive • Fluorescent or radioactive reporter labeling is required	26
Alkaline gel electrophoresis	Tenths of fmol		• Detection is limited to strand breaks	46
Immunoassay coupled with CE-LIF	3×10^{-21} moles	• Selective to specific type of DNA damage • Sensitive detection	• Fluorescent labeling is required • Cross reactivity of antibodies • Expensive Ab synthesis	61
Molecular Beacon Probes	DNA MB: 4 picomol Ch MB: 0.8 picomol	• Simple • Selective to the overall damaged bases	• Not selective to specific damage types. • Labeling with fluorescent reporter and quencher is required • Expensive synthesis	80
2AP probe	1 picomol	• Simple • Selective to the overall damaged bases	• Not selective to specific damage types • Synthesizing specific DNA probes with 2AP bases • Expensive synthesis	82

Tb^{3+}/hairpin probe	0.4 picomol	• Sensitive • Low cost • Selective to overall amount of damage • Simple • No need for fluorescent reporter attachment	• Not selective to specific damage	83
Mass spectrometry	20-50 fmol	• Selective detection	• Several sample pretreatment steps • Expensive instrumentation	13
^{32}P post-labeling	Hundreds of attomoles	• Sensitive detection	• Several sample pretreatment steps • Elaborate • Time consuming • Poorly informative • Lack of calibration	12
Ac voltammetry	1 fmol	• Sensitive detection	• Detection is limited to strand breaks	14

*The limit of detection in moles is used to represent the sensitivity of the technique.

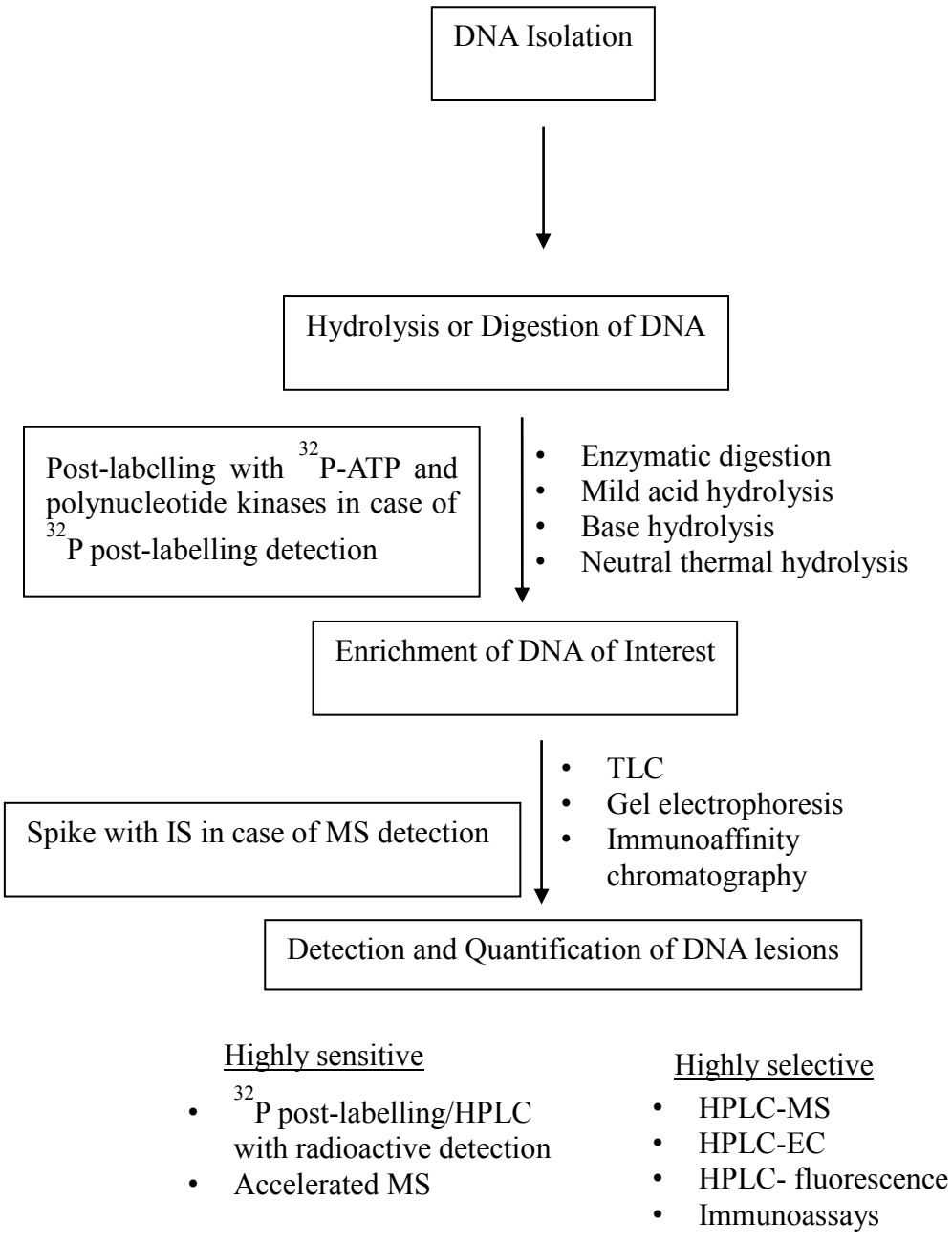
DNA Isolation
Hydrolysis or Digestion of DNA
Post-labelling with ^{32}P-ATP and polynucleotide kinases in case of ^{32}P post-labelling detection
• Enzymatic digestion
• Mild acid hydrolysis
• Base hydrolysis
• Neutral thermal hydrolysis
Enrichment of DNA of Interest
Spike with IS in case of MS detection
• TLC
• Gel electrophoresis
• Immunoaffinity chromatography
Detection and Quantification of DNA lesions
Highly sensitive
• ^{32}P post-labelling/HPLC with radioactive detection
• Accelerated MS
Highly selective
• HPLC-MS
• HPLC-EC
• HPLC- fluorescence
• Immunoassays

Figure 1.

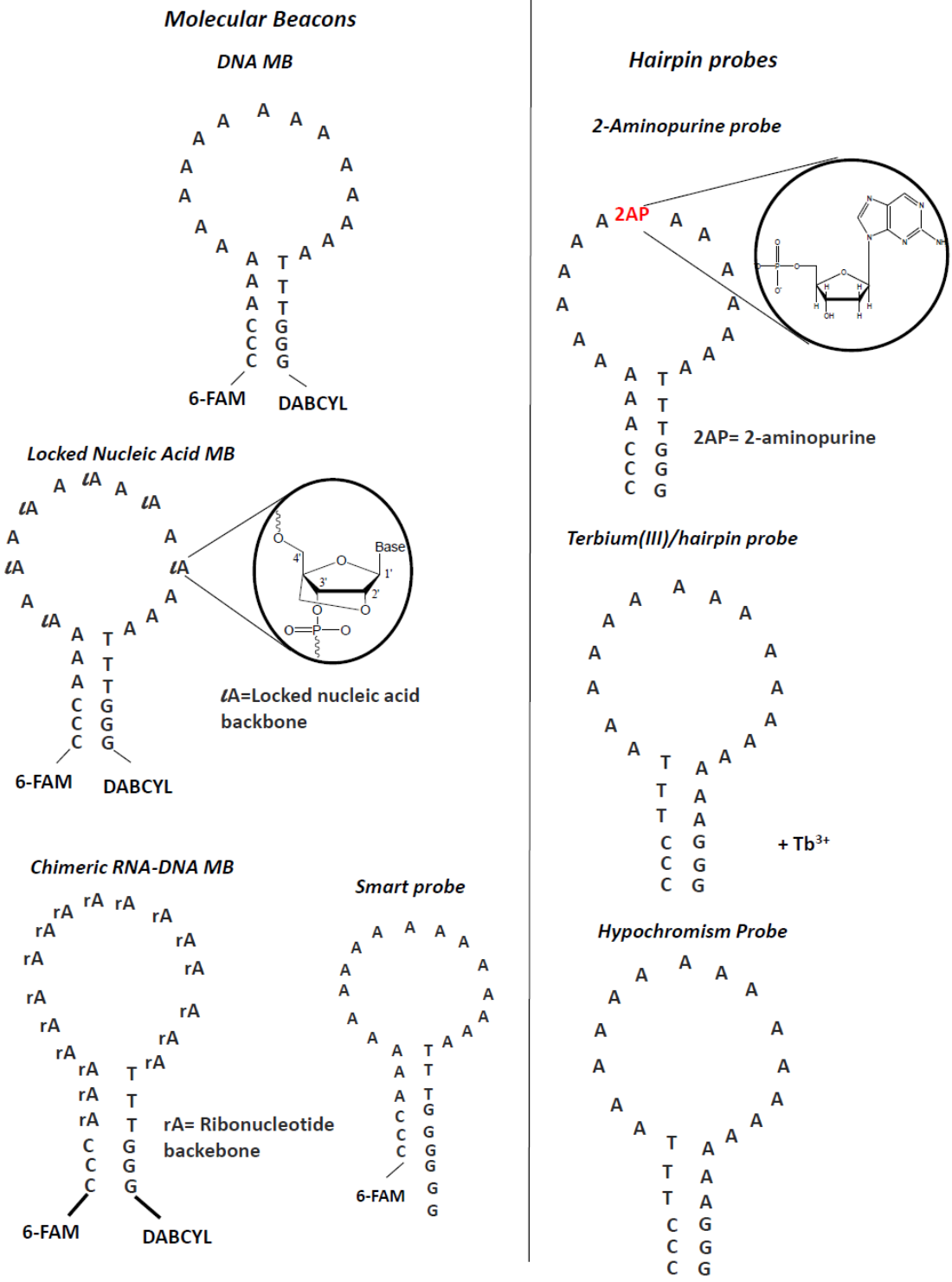
Molecular Beacons
DNA MB
6-FAM
DABCYL
Locked Nucleic Acid MB
ℓA=Locked nucleic acid backbone
6-FAM
DABCYL
Chimeric RNA-DNA MB
rA= Ribonucleotide backebone
6-FAM
DABCYL
Smart probe
6-FAM
Hairpin probes
2-Aminopurine probe
2AP
2AP= 2-aminopurine
Terbium(III)/hairpin probe
+ Tb3+
Hypochromism Probe

Figure 2.

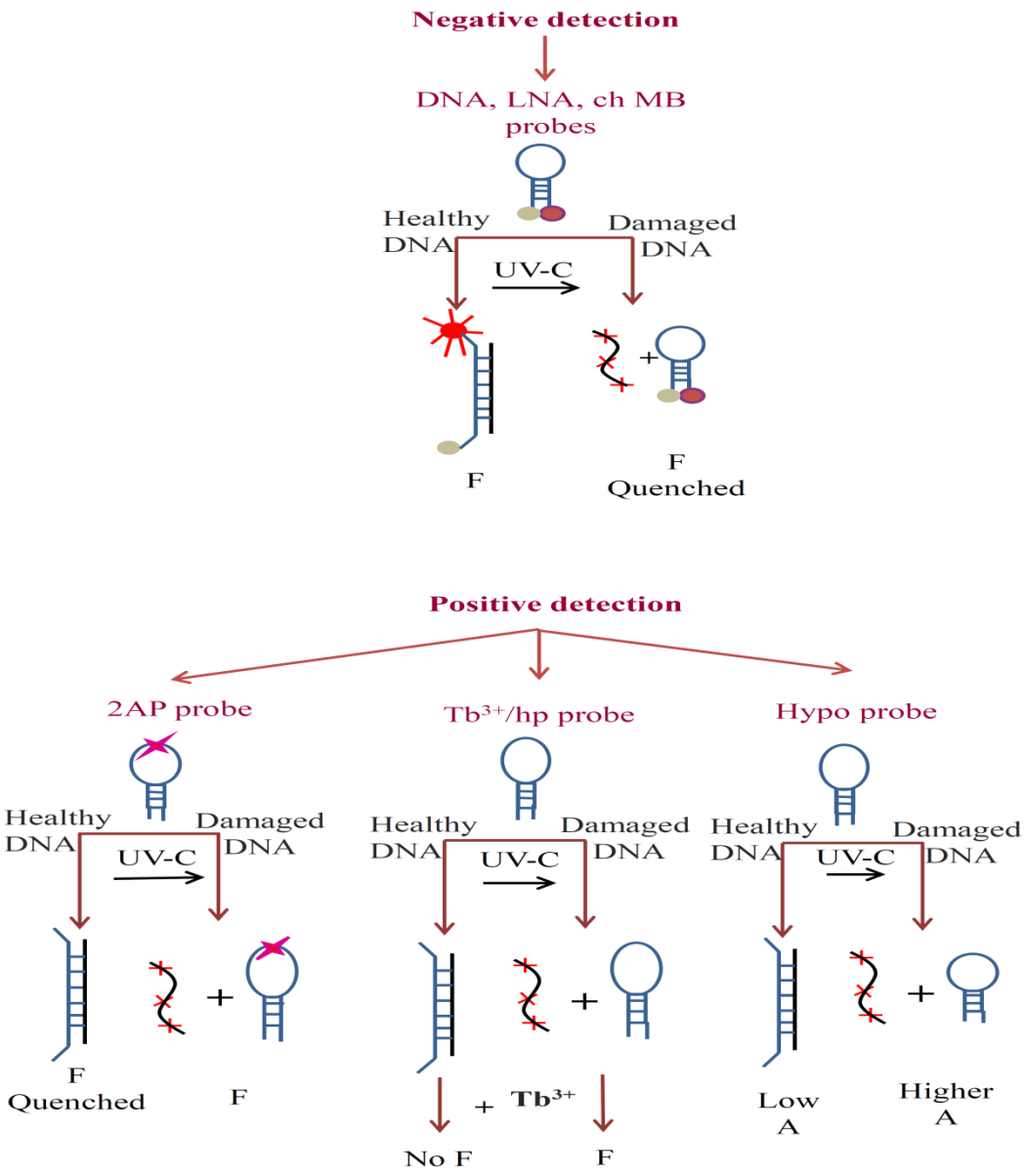
Negative detection
DNA, LNA, ch MB probes
Healthy DNA
Damaged DNA
UV-C
F
F Quenched
Positive detection
2AP probe
Tb3+/hp probe
Hypo probe
Healthy DNA
Damaged DNA
UV-C
F Quenched
F
+ Tb3+
No F
F
Low A
Higher A

Figure 3.