Published in final edited form as:

Nanomedicine. 2006 June ; 2(2): 74–81. doi:10.1016/j.nano.2006.04.004.

Modifying the Function of DNA Repair Nanomachines for Therapeutic Benefit

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Abstract

This article, which is based on a presentation at the First Annual Meeting of the American Academy of Nanomedicine, is divided into three parts. In the first, we describe naturally occurring DNA repair nanomachines, using the nanomachine that executes the nonhomologous end-joining (NHEJ) reaction for DNA double-strand break (DSB) repair as an example. In the second, we discuss therapeutic benefits that may be derived from the ability to modify the behavior of naturally occurring nanomachines, using as an example the concept of delaying DSB repair in rapidly dividing cancer cells to increase their natural sensitivity to radiation therapy. In the third part, we discuss similarities in the overall size, shape, and design of different nanomachines that manipulate DNA and RNA, and the possibility of developing nanomachines with new specificities not found in nature.

Naturally occurring DNA repair nanomachines

DNA repair nanomachines

The human cell nucleus is a membrane-bounded organelle with a typical diameter of 5,000 to 10,000 nm. The nucleus is crowded with nanometer-scale structures associated with specific functions such as RNA transcription, RNA processing, DNA replication, and DNA repair. Unlike the organelles of the cytoplasm, these structures are not enclosed within individual lipid membranes; rather, they are self-organizing nanomachines.

DNA repair occurs continuously in human cells, reflecting a response to unavoidable damage from environmental agents and endogenous oxidative species. The human genome encodes about 150 genes for DNA repair proteins. These proteins organize to form about a dozen different types of repair nanomachines, each specializing in a different type of damage.

DNA double-strand breaks and the maintenance of genome integrity

DNA double-strand breaks (DSBs) are a rare type of damage with unusually potent biological effects [1]. Because each human chromosome is composed of a single DNA molecule, double-strand breaks lead to chromosome breaks, and thus to production of acentric chromosomal fragments and other aberrations. Even one unrepaired DSB can lead to large-scale loss of information during cell division and consequently, cell death [2]. Exposure to ionizing radiation is a principal natural cause of DSBs. Passage of a high-energy photon or other particle through tissue generates reactive chemical species that are distributed inhomogeneously along

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Competing interests: Intellectual property described in this article has been licensed by the Medical College of Georgia to Apeliotus Technologies, 1456 North Morningside Drive, NE, Atlanta GA 30306. WSD is a consultant for Apeliotus Technologies.

Material in this article was originally presented at the First Annual Meeting of the American Academy of Nanomedicine, Baltimore, MD, August 15-16, 2005.

nanometer scale tracks. When a radiation track intersects the DNA helix, both strands are damaged simultaneously, which often results in the formation of a DSB.

DSB repair begins almost immediately after damage is detected, with a rapid component having a half time of 7-14 min and a slow component having a half time of 60-90 min [3]. The speed with which DSB repair occurs reduces the amount of time that a cell with a broken chromosome spends in this critically vulnerable state. Two different types of nanomachines function to repair DSBs. One of these carries out a process of repair based on homologous recombination, using an existing undamaged sequence, such as a sister chromatid, as a template [4]. The other carries out repair based on nonhomologous end joining (NHEJ), where DNA ligase directly rejoins broken DNA ends that have little or no homology, often leaving a small region of altered sequence at the break site [5,6]. NHEJ appears to be the main pathway at clinically relevant doses of radiation, and is therefore the focus of this article.

Structure, function, and assembly of the NHEJ nanomachine

NHEJ takes place within visible, subnuclear structures known as repair foci, or alternatively, ionizing radiation-induced foci. These structures are defined by the presence of a domain of modified chromatin enriched with a phosphorylated histone variant, γ -H2AX, which can be visualized with anti- γ -H2AX antibodies [7]. Foci appear within minutes after irradiation and disappear within 60-90 min, matching the tempo of the repair process itself. Moreover, the number of foci corresponds closely with the number of DSBs anticipated at a given dose of radiation. Fig 1A shows an example of human melanoma cells that were irradiated with a 0.1 Gray (Gy) dose, which is predicted to generate approximately 3 DSBs per diploid human genome. Indeed, 3-4 foci are visible in these cells 30 min post irradiation. At 90 min post-irradiation, most of the foci have disappeared.

The internal structure of the repair foci remains unknown, although we can deduce some information based on genetic, biochemical, and cytologic studies (Figs. 1B, 1C). Using immunofluorescence microscopy, repair foci appear initially as diffraction-limited point sources, suggesting that their maximum dimension is no more than 250 nm (Fig. 1B). The domain of γ -H2AX-containing chromatin extends, on average, 1 megabase pair (Mbp) to either side of the break [7]. A DNA region of this size has a contour length of about 70,000 nm, several hundred times the maximum estimated diameter of repair foci. The DNA in the foci therefore must be highly compacted. It has recently been proposed that γ -H2AX may function as an anchor to hold broken chromosomal ends in proximity [8]. As such, γ -H2AX recruits many of the other proteins associated with repair foci.

Repair foci contain a number of proteins in addition to γ -H2AX, including 53BP1 [9], MDC1 [10], phosphorylated Chk2 [11], HDAC4 [12,13], and the Mre11•RAD50•NBS1 complex [14,15]. Because these proteins can be readily visualized by indirect immunofluorescence, which generally does not have the sensitivity to detect fewer than several dozen copies of a given protein, each must be present in multiple copies. Interestingly, these proteins are mostly involved in cell-cycle or other regulatory functions, and none (with the possible exception of the Mre11•Rad50•NBS1 complex) has an enzymatic activity that is essential for NHEJ in mammals. The lack of biochemical activities directly related to repair, as well as their abundance, suggests that many of the proteins observed cytologically in repair foci make up the structural matrix of the foci, rather than the enzymatically active core. Whether this matrix has an ordered structure, or is more of a nonspecific nucleoprotein aggregate, has not been settled.

Whereas cytologic characterization of repair foci provides a “top-down” approach to understanding NHEJ, genetic and biochemical studies provide a “bottom-up” approach, focusing on the role of individual enzymes and other DNA-binding proteins. The model in Fig.

1C summarizes our present understanding of the core NHEJ complex based on these studies. The two halves of the broken DNA are shown as double helices entering from opposite directions. Protein-DNA contacts detected by photo-crosslinking are shown as circles on the DNA helix [16,17]. Five gene products are known to bind directly to broken DNA ends or to have a direct enzymatic role in NHEJ. Ku protein, a heterodimer of 70 kDa (Ku70) and 80 kDa (Ku80) subunits, binds avidly to broken DNA ends [18], translocates inward after binding, and recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [19,20]. The size and shape of Ku70 and Ku80 are based on a high-resolution structure, which reveals DNA passing through a hole in the center of a doughnut-shaped heterodimer [21]. The size ($9 \times 6 \times 2$ nm) and shape of DNA-PKcs is based on single particle reconstruction studies [22]. DNA-PKcs sequesters and protects the DNA ends and is released by autophosphorylation [23], probably triggered by stable pairing of opposing DNA ends [24]. Autophosphorylation of DNA-PKcs allows other repair proteins to access the DNA termini [25,26]. A complex of DNA ligase IV and XRCC4 carries out the chemical steps of ligation. Biochemical evidence suggests that these proteins form a mixed tetramer [27]. The tetramer is presumably asymmetric, as only one DNA ligase IV molecule can be seen to contact the XRCC4 dimer in a partial crystal structure of the complex [28]. The position of the DNA ligase IV•XRCC4 complex, relative to other components, is unknown and has been omitted from the figure.

Of the five gene products believed to make up the core enzymatic machinery for NHEJ, only a phosphorylated form of DNA-PKcs has been demonstrated to be present in repair foci by immunofluorescence [29]. Although the other gene products must be present in order for repair foci to function, it may be that localization of one or two copies of these proteins within the foci is not readily evident against the overall background of nuclear staining.

Modifying the function of DNA repair nanomachines for therapeutic benefit

Human exposure to ionizing radiation

Human exposure to ionizing radiation comes from terrestrial sources, such as naturally occurring radioisotopes (^{222}Rn , ^{40}K , ^{14}C , ^3H), and from cosmic sources, including solar protons and galactic cosmic radiation. Together, these sources of radiation account for an exposure of 3 milliSievert annually (mSv; 1 mSv=1 mGy for X-rays and gamma rays). For many people, exposure from anthropogenic sources vastly exceeds this natural background. Medical diagnostic procedures, especially nuclear medicine and CT scans, are associated with exposure on the order of 5-50 mSv. Cancer radiotherapy, administered to 750,000 patients annually in the United State alone, is associated with exposures, limited to the tumor region, on the order of 50-75 Sv, or 25,000-fold above the natural background.

Modifying the function of DNA repair nanomachines in radiotherapy patients

An ability to modify the function of DNA repair nanomachines in radiotherapy patients would bring an obvious benefit. As an example, lung cancer is the most common cancer in both men and women in the US, and only 30% of cases are operable for cure at the time of presentation [30]. Radiotherapy is the primary mode of treatment for the remaining cases, but its ability to cure lung cancer is severely limited by the susceptibility of normal lung tissue to radiation-induced damage. Although there is no doubt that better tumor control can be achieved with higher radiation doses, radiation levels that can be tolerated by normal lung tissue are generally insufficient to eradicate the cancer [31]. NHEJ appears to be important in determining levels of tumor radioresistance. Particularly striking evidence has been seen in a study of multiple human lung cancer cell lines, where radioresistance was directly proportional to the level of the NHEJ protein, DNA-PKcs [32].

How might the ability to affect NHEJ increase the efficacy of radiation therapy? Clearly, if one could deliver a repair inhibitor specifically to tumor cells, and not normal cells, one could achieve better tumor control at a given dose, without increasing the risk of harm to normal tissue. We hypothesize, however, that even without a specific delivery mechanism, modification of the NHEJ nanomachine could lead to therapeutic gain. Tumor cells are intrinsically more sensitive to radiation than normal tissue because they divide rapidly and because they are compromised with respect to DNA damage-dependent cell-cycle checkpoints. Modifying the DNA repair machine to introduce a time lag in the repair process could potentiate this intrinsic sensitivity by increasing the probability that the tumor cell will divide prior to repair, leading to irreparable loss of genetic material and thus to clonogenic death. We hypothesize that normal cells, because of their slower rate of division and intact checkpoints, should be less affected by a delay in repair.

Single chain antibodies as a platform for modifying nanomachine function

We have explored the use of single-chain antibody (abbreviated as scFv, for single-chain fragment, variable) technology as a platform for modifying nanomachine function. Naturally occurring antibodies contain multiple antigen-binding sites, formed at the interface of separate heavy and light polypeptide chains, grafted onto a large, invariant structural framework [33]. ScFvs are recombinant molecules containing a single antigen binding site and only a minimal portion of the invariant framework. As the name suggests, the heavy and light chains are connected, via a flexible linker, to form a single polypeptide chain that can be readily expressed in recombinant form. Single-chain antibodies are more “drug-like” than the parent antibodies from which they are derived. A scFv has only about 20% of the mass of an immunoglobulin G molecule (the most common type of naturally occurring antibody), facilitating uptake by cells and entry into the nucleus. In addition, because they are recombinant molecules, scFvs can be genetically modified or chemically derivatized to add desired functionalities.

A single chain antibody directed against DNA-PKcs (scFv 18-2)

A single-chain antibody (scFv 18-2) was derived from a parent monoclonal antibody previously shown to bind to DNA-PKcs and to partially inhibit kinase function [34]. The epitope recognized by scFv 18-2 was mapped by immunoreactivity with successively smaller regions of the protein obtained by proteolytic cleavage, *in vitro* transcription-translation, and chemical synthesis. These studies showed that scFv 18-2 recognizes a 25-residue peptide near the center of the 4127-residue DNA-PKcs molecule [35].

The significance of this location became apparent only recently. A very extensive modeling effort, drawing on a variety of sources, has allowed the mapping of the primary sequence of DNA-PKcs into the electron density model obtained from single-particle reconstruction [22]. It appears that the scFv 18-2 epitope lies in an extended arm domain connecting a large DNA binding “palm” region, at the N terminus, with a catalytic head, at the C terminus. The arm undergoes a large conformational change upon DNA binding [36]. We hypothesize that the ability of scFv 18-2 to block or delay NHEJ may be linked to its ability to block this conformational change (Fig. 2).

Inhibition of NHEJ by scFv 18-2 *in vitro* and *in vivo*

Evidence that scFv 18-2 interferes with NHEJ is based on three findings:

- scFv 18-2 inhibits DNA end joining in a cell-free system containing linearized plasmid substrate, HeLa cell nuclear extract, and recombinant DNA ligase IV/XRCC4 [35].
- Microinjection of scFv 18-2 into telomerase-immortalized human retinal pigment epithelial (RPE) cells expressing the adenovirus E1A oncoprotein [37], which are a

model for early-stage cancer, greatly increased the toxicity of a single, relatively low dose (200 cGy) of ionizing radiation [35]. This dose is approximately equal to a single clinical radiotherapy fraction.

- Microinjection of scFv 18-2 into human melanoma cells markedly prolonged the lifetime of ionizing radiation-induced repair foci [35], suggesting that it acts directly to introduce a time lag in the DNA repair process – the desired behavior for a clinically useful radiosensitizer. Figure 3, taken from ref [35], illustrates this ability to prolong the lifetime of repair foci.

Together, these data suggest that single-chain antibodies can provide a useful technology platform for modifying the function of the NHEJ nanomachine. A particular advantage of scFv 18-2 is its high target specificity. The sequence in the epitope region is unique to DNA-PKcs, not present in other members of the same protein kinase family (the phosphatidylinositol 3-kinases). In this respect, it differs from small-molecule inhibitors directed against the phylogenetically conserved kinase active site [38,39]. Indeed, scFv 18-2 has no mechanism of action in cells that are outside a radiation field, suggesting that systemic toxicity will be minimal if administered as a radiosensitizer.

The next challenge in development of scFv 18-2 will be to address the problem of delivery to its intracellular target. Although single-chain antibodies are more “drug-like” than their parent monoclonal antibodies, they do not efficiently penetrate the cell membrane. We anticipate that this problem may be overcome in a variety of ways, including the use of liposomes and other nanoparticle carriers. A general review of nanoparticle-based drug delivery is beyond our present scope. We note, however, that elegant recent studies have demonstrated the value of cell-penetrating and nuclear localization peptides in promoting delivery of nanoparticles to intracellular compartments [40]. Conjugation to biofunctionalized particles offers a promising path for further development of the scFv platform.

Common design principles in nanomachines for RNA synthesis and DNA repair

The NHEJ nanomachine as a model for other processes

The NHEJ nanomachine provides an attractive model for studying other nuclear processes, including other types of DNA repair, RNA transcription, and RNA processing. Attributes of the NHEJ nanomachine that make it an attractive model include:

- Well-characterized components. Five polypeptides account for core function. Atomic-resolution structures are available for two of these (Ku70 and Ku80) [21], a main-chain model is available for a third (DNA-PKcs) [41], and partial structures for a fourth (XRCC4) [28,42]. All five components have been well studied genetically.
- Relative simplicity. Efficient, phosphorylation-regulated DNA end joining can be reconstituted in reactions containing just seven polypeptides (the five core polypeptides and one additional heterodimeric repair factor) [43]. Although repair foci formed *in vivo* contain additional proteins, the complexity appears manageable, especially in comparison to the 150 or more protein and RNA components believed to be present in the spliceosome [44] and in the nucleolus [45], structures responsible for mRNA processing and ribosomal RNA biogenesis, respectively.
- Inducible assembly. Unlike nanomachines for RNA synthesis, the NHEJ nanomachine is assembled only in response to a well-defined stimulus. It is thus possible to study not only its steady-state behavior but also its assembly and disassembly.

Are there common design principles for DSB repair and RNA synthesis nanomachines?

There is evidence for interrelationship of transcription and DSB repair. Nuclear extracts from cells lacking Ku protein, for example, show a characteristic defect in transcriptional reinitiation, and certain Ku mutants have a dominant negative effect on transcription [46]. Ku can be chemically crosslinked *in situ* to regions of actively elongating chromatin [47]. Indeed, for reasons not clearly understood, loss of Ku function in human somatic cells leads rapidly, within a few days, to loss of cell viability. This loss of viability does not appear to reflect accumulation of DNA defects, and the underlying cause remains under investigation. We hypothesize that NHEJ nanomachines may assemble on or near the surface of “active chromatin hubs” that serve as the main site of pre-mRNA production in the cell; the ability of these hubs to organize chromatin into loops may stabilize pairing of broken DNA as the repair machine assembles.

Although machinery required for mRNA biogenesis is vastly more complex than the machinery for NHEJ, both processes require the same elementary steps, including recognition of DNA sequences and structures, self-assembly of protein components into a nanoscale structure, and synthesis of internucleotide phosphodiester bonds. The core process of transcript elongation is carried out by the enzyme RNA polymerase II, a multisubunit enzyme with aggregate molecular weight on the same order as the core NHEJ machine. Indeed, structural models [41,48] reveal overall similarities in size, shape, and design (Fig. 4). In both cases, DNA substrate feeds into a cavity created by the protein. In the case of NHEJ, free DNA ends are positioned for further processing, whereas in the case of RNA polymerase II, the strands are continuous, with single strands exiting through pores (not shown).

Development of nanomachines with novel biological functions

There has recently been a great deal of interest in selective incision, resection, and repair of the genome. Engineered synthetic nucleases have been shown to be capable of cutting DNA at selected sites, promoting targeted repair of inborn sequence errors [49]. The difficulty is that the reactions are rather promiscuous. Nucleases are only partially specific for their target sequences, and the free ends generated by nuclease incision are highly intrinsically susceptible to incorrect rejoining. Nature has faced much the same problem in designing a system for targeted recombination at specialized sites in the immune system. In the case of V(D)J recombination, the RAG1•RAG2 nuclease creates the incision, but the general NHEJ machinery is recruited, apparently in a concerted reaction, to rapidly and accurately reseal the breaks [50].

One can envision co-opting the NHEJ machine for similar purposes artificially. Thus, an artificial nuclease could be engineered to recruit NHEJ proteins and cleave DNA only in the context of a repair focus. DNA ends would be sequestered and protected from incidental damage and from rejoining in unwanted combinations. Such a machine could help realize the promise of gene therapy without the problems accompanying today's approaches.

Acknowledgments

We thank John Edwards of Apeliotus Technologies and colleagues at the Medical College of Georgia for helpful discussions. We thank Dr. Rhea-Beth Markowitz for editorial support. A number of the citations in this article are to other reviews, and we apologize to the authors of many valuable papers from the primary literature that could not be cited because of space constraints.

This work was supported by U.S. Public Health Service awards number GM 35866 and CA 98239 and by the U.S. Department of Energy Low Dose Radiation Research Program award number DE-FG02-03ER63649. WSD received additional support as an Eminent Scholar of the Georgia Research Alliance.

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Figure 1.

DNA repair foci. A. Visualization of repair foci by fluorescence microscopy. Human melanoma cells were mock-irradiated with 0 Gray (Gy) of ionizing radiation (panels a, d) or irradiated with 0.1 Gy and allowed to recover for 30 minutes (panels b, e) or 90 minutes (panels c, f). Cells were fixed and stained with DAPI to show position of nuclei (panels a, b, c) and mouse monoclonal anti- γ -H2AX antibody and AlexaFluor 594-conjugated anti-mouse IgG secondary antibody to show repair foci (panels d, e, f). Each panel shows a single cell nucleus. Filled arrow in panel e highlights 3-4 repair foci. Open arrows in panels d, f indicate nuclei without foci. Scale bar, 10 μm . B. Hypothetical structure of repair focus at 500 $\times$ greater magnification than in A. Complex of Ku, DNA-PKcs, and DNA ends is shown approximately

to scale at the center of the focus. Chromatin and signaling proteins account for remaining mass, shown here as undifferentiated background (see text). C. Structure of core NHEJ complex at $20\times$ greater magnification than in B. Ku heterodimer (Ku70 and Ku80, black and dark grey respectively) binds to broken DNA ends and recruits DNA-PKcs (lighter grey). Size, shape, and relative positions of proteins are based on structural data (see text). Protein-DNA contacts detected by photo-crosslinking are shown as circles on the DNA helix. Data in panel A are adapted from ref [35]; cells shown here were microinjected with a control single-chain antibody variable fragment (scFv) that does not interfere with survival.

A. Epitope mapping

B. Hypothetical mechanism of action of scFv 18-2

Figure 2.

Epitope mapping and mechanism of action of scFv 18-2. A. Diagram shows location of fragments and peptides used for epitope mapping relative to kinase catalytic domain. For details and supporting data, see ref [34]. Epitope was mapped to an N-terminal Caspase 3 fragment by immunoblotting. Epitope was further mapped to fragments spanning residues 1734-2228 and 1901-2081 by immunoprecipitation of polypeptides expressed by coupled transcription-translation in the presence of ^{35}S -methionine. Epitope was finally mapped to peptide spanning residues 2001-2025 by ELISA and surface plasmon resonance binding studies. B. Hypothetical mechanism of action of scFv 18-2 in blocking NHEJ. According to a structural model based on single-particle reconstruction, scFv 18-2 epitope lies in an extended arm domain connecting a large DNA-binding “palm” region (N terminus) with a catalytic head (C terminus). The arm domain undergoes a large conformational change upon DNA binding. We hypothesize that the binding of scFv 18-2 to the extended arm blocks the conformational change and inhibits the DNA binding, resulting in inhibition of the NHEJ pathway.

Figure 3.

ScFv 18-2 prolongs the lifetime of γ -H2AX foci. SK-MEL28 melanoma cells were co-injected with a reporter plasmid, pEGFP-N1, and either scFv 18-2 or a control single-chain antibody, scFv 147. Cells were irradiated at 1.5 Gy to induce approximately 45 DSBs per genome. Control cells were mock-irradiated with 0 Gy. Cells were fixed at 30 min or 90 min after treatment and stained with anti- γ -H2AX antibody (panels a-g), anti-GFP antibody (panels h-n) or DAPI (panels o-u) as described in ref [35]. GFP is a marker that indicates which cells were successfully microinjected; DAPI stains DNA and reveals nuclei of all cells in field. Scale bar indicates 10 μ m. Data are taken from ref [35].

A. NHEJ complex

B. RNA polymerase II complex

Figure 4.

Similar size, shape, and design of nanomachines for NHEJ and pre-mRNA synthesis. A. Ku•DNA-PKcs•DNA complex, drawn as in Figure 1. B. RNA polymerase II complex, styled after [48]: Duplex DNA enters the main cleft formed in the RNA polymerase II transcribing complex. Both NHEJ and RNA synthesis require the same elementary steps, including recognition of DNA sequences and structures, self-assembly of protein components into a nanoscale structure, and synthesis of internucleotide phosphodiester bonds.