Changes known as epigenetic modifications play an important role in cancer development. Being able to analyze them quickly and reliably could contribute significantly to the further development of personalized therapy. A research team from the Institute of Physiology at the University of Freiburg has now succeeded in characterizing the chemical changes in proteins that are typical for epigenetic modifications using nanopore analysis. The researchers have published their research results in the *Journal of the American Chemical Society* (*JACS*).

In recent years, nanopores have become a widely applicable tool for the analysis of molecules. Due to their special properties, they allow the structure of molecules to be analyzed within fractions of a second: As cylindrically arranged proteins, nanopores form tiny channels only a few millionths of a millimeter (nanometer) in diameter that can be embedded in biomembranes.

"For the experiments, we apply a constant voltage across the membrane so that ions from the surrounding medium flow through the pore. This creates a constant, precisely measurable electric current," explains Prof. Dr. Jan C. Behrends from the Faculty of Medicine at the University of Freiburg, in whose laboratory the now-published experiments took place. However, when a molecule migrates into the pore, the current is blocked: the larger the molecule, the more strongly it is blocked too.

**A protein in the research spotlight: H4**

In the context of the experiments now published, the Freiburg scientists devoted themselves to the investigation of the so-called histone protein H4. This protein is firmly associated with DNA in all cells with a nucleus and is one of the best-researched targets of epigenetic modifications. A region at the N-terminal end of the protein is particularly affected by these modifications.

"The protein sequence there contains the amino acid lysine several times," Behrends explains. Acetyl or methyl groups, for example, can be attached to these lysines, which are designated K8, K12 and K16 according to their position in the protein chain, as part of epigenetic modifications. Which chemical modification takes place at which lysine position is definitely of medical importance, as the Freiburg physiologist points out. "Acetylation
at K16, for example, is important for human
development, while methylation at K12 plays a role
in the development of some prostate and lung
tumors, according to the latest results from Medical
Center—University of Freiburg."

**Detecting changes with the help of a nanopore**

In their experiments, Behrends and his team were
now able to clearly distinguish H4 fragments with or
without acetylation, as well as fragments with one,
two or three acetylations. Moreover, they
succeeded in demonstrating that the nanopore they
used was also sensitive to the site of acetylation:
histone fragments with an acetyl group at K8
blocked current through the pore more strongly
than those acetylated at K12, and these in turn
more strongly than those with a K16 acetylation.

"This kind of sensitivity is surprising in that these
fragments are identical in terms of their mass and
total volume," Behrends says. Thus, the pore
current appears to be sensitive not only to the size,
but also to the shape of the molecule. It was
equally easy to distinguish between the different
variants of doubly acetylated histone fragments—K8
and K12, K8 and K16, and K12 and K16—again,
despite the identical mass. H4 fragments
methylated to different extents and at different
positions also blocked the current through the pore
to different degrees, although not as clearly as the
acetylated variants.

"We have been able to show for the first time
through our experiments that nanopore analytics
allows us to distinguish molecules not only by their
size, but also by their shape," says study leader
Behrends. Molecular dynamics simulations
conducted by the research group led by Aleksei
Aksimentiev from the University of Illinois in the
U.S.—also involved in the study—and show that a
highly inhomogeneous electric field inside the pore
plays a key role for this effect.

**Future vision: Optimized medical diagnostics**

While the sequencing of DNA using nanopores is
already established and commercialized, the
development of nanopore-based analysis of
proteins is just beginning, Behrends says. "The
difficulty with sequencing proteins is that these are
molecules with very non-uniform charge patterns."
While DNA, which is negatively charged, migrates
directionally in the electric field and can thus be
pulled through the pore base by base, proteins
consist of building blocks made of the amino acids
with different charges. As a result, directed
movement in the electric field and "scanning" amino
acid by amino acid is not possible. The Freiburg
scientists therefore relied on a different approach
for their experiments. Instead of a pore with a short
constriction, as used in DNA sequencing, they used
a tailor-made pore with a kind of molecular trap.
"This allowed the entire protein fragment to be
captured at once," says Behrends.

It is not yet clear up to which fragment size this type
of analysis can be used. However, additional
experiments show that the method will also be
suitable for the analysis of the H4 fragments
previously used in epigenetic research. These
contain 14 amino acids instead of the ten used
here, and are currently investigated for epigenetic
modifications with tandem mass spectrometry, a
highly elaborate technique. The researchers hope
that the nanopores will make the analysis much
simpler, faster and more cost-effective, and that it
can be carried out close to the patient.

The further development of nanopore analysis of
proteins for medical diagnostics and its
implementation in concrete products and services
is also one of the central projects of the recently
approved BMBF Cluster4Future nanodiagBW,
which Behrends heads together with Prof. Dr. Felix
von Stetten of the Hahn-Schickard-Gesellschaft,
which is the lead for this project.

**More information:** Tobias Ensslen et al,
Resolving Isomeric Posttranslational Modifications
Using a Biological Nanopore as a Sensor of
Molecular Shape, *Journal of the American
Chemical Society* (2022). DOI:
10.1021/jacs.2c06211

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