Ageing is a complex process that is greatly influenced by environmental and genetic factors. Ageing at the cellular and molecular level is characterized by the accumulation of post-translationally modified proteins that are derogative to cells, termed Degenerative protein modification (DPM). A particular class of DPM, occurring under the impact of urea, termed brain ureido degenerative post-translational modifications (uDPM) has also been implicated in the molecular pathogenesis of Alzheimer's disease and related neurodegenerative disorders. These modifications can disrupt normal protein functions by altering their conformation, enzymatic activities, receptor recognitions, and physiological capabilities. Carbamylation is an age-related uDPM that results from the non-enzymatic modification of amino groups of ε-amino groups on lysine in proteins. The significance of carbamylation in some age-related disorders such as cataracts, rheumatoid arthritis, and cardiovascular diseases is well documented. However, the role of carbamylation in the pathogenesis of various neurodegenerative disorders, which are also age-dependent, has not been explored much. The aim of the current article is a retrospection of carbamylation, its effect on proteins, and its implications in ageing and neurodegenerative diseases.

**Keywords:** Age-related disorders, Carbamylation, Neurodegenerative diseases, Post-translational modifications

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**Abbreviations:** AGE, Advanced Glycation End Products; CDP, Carbamylation-derived Products; CML, N-ε-carboxymethyl-lysine; KCNO, Potassium cyanate; MBPT, Microtubule Binding Protein Tau; MPO, Myeloperoxidase; NDs, Neurodegenerative Disorders; NEPTM, Non-Enzymatic Post Translational Modifications; PTM, Post Translational Modifications; TDP 43, TAR DNA-binding protein 43; TEM, Transmission Electron Microscopy; uDPM, Ureido Degenerative Post-Translational Modifications

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Carbamylation is one such modification, corresponding to isocyanic acid binding to the proteins. Carbamylation of proteins was first described some decades ago in vivo and was a well-known protein chemical reaction⁷,⁸; however, its involvement in the pathophysiology across humans and in various diseases has been researched recently⁹.

This review focuses on the relationship between carbamylation and neurodegenerative disorders and provides an overview of the process of carbamylation, its contribution towards molecular ageing of protein, and the involvement of carbamylation in neurodegeneration and other diseases associated with ageing.

### Carbamylation

Carbamylation is a NEPTM defined by the non-enzymatic binding of "carbamoyl moiety" (-CONH2) to the free amino functional group of peptides, proteins, and free amino acids. The primary interaction happens between an electrophile (isocyanic acid) and a nucleophile functional group (α-NH₂ of amino acid or ε-NH₂ of Lysine side chain). The by-products formed by these reactions are termed carbamylation-derived products (CDP)¹⁰.

While carbamylation may occur at other functional groups such as the cysteine sulfhydryl group, phenolic group of tyrosine, imidazole group of histidine, or a carboxyl group present in aspartic and glutamic acid, however, at the physiological pH, homo-citrulline (HCit) and α-carbamyl amino acids are the only stable amino acids¹¹-¹³.

In vivo, carbamylation modification is primarily affected by isocyanic acid, formed by the spontaneous dissociation of urea (which is reversible) into cyanate and ammonia in aqueous conditions. The tautomeric form of cyanate is isocyanic acid and its formation is temperature and pH dependent¹⁴. Isocyanic acid is a very reactive electrophile that quickly reacts with nucleophiles and modifies the α-amino group of primary amines, ε-amino group of lysine, and free sulfhydryl group of free amino acid, peptides, and proteins. The carbamylation reaction rate depends on the pKa value of the group and increases with decreasing pKa. Adding the carbamoyl group (-CONH₂) to the amino functional group in protein cause a change in the electrical charge of the protein. If added to the Lysine, the transformed product is termed homo-citrulline, which causes a change in molecular weight and function. The final by-product of urea is formed after human nitrogen metabolism and has been considered an unreactive waste product⁶,¹⁵,¹⁶.

The reaction may also happen from another source of isocyanic acid: the enzymatic action of myeloperoxidase (MPO) on thiocyanate with hydrogen peroxide present in system. The source of thiocyanate can be from diet or various environmental factors such as smoking and diet contained in certain vegetables and fruits like cauliflower, brussels sprouts, some dairy products, and roots like cassava¹⁷. MPO, a heme-containing enzyme, is also known for its abundance in inflammatory cells like monocytes, macrophages, and neutrophils, transforming thiocyanate into hydroxocyanic acid and cyanate¹⁸.

Figure 1 represents the major biochemical pathway of carbamylation, isocyanic acid formation and isocyanic acid reaction with the protein amino group. Inclusive of the MPO pathway and urea deamidation pathway, many other environmental sources of isocyanic acid formation exists that can cause the reaction. Usage of biofuel, cooking gas, tobacco smoke, industrial contamination, etc., contains cyanate that can be inhaled from urban air¹⁸. Casava roots are one such indirect food source of cyanate; it contains cyanogen that metabolizes to cyanate after its consumption. A group of clinicians has performed research on 40 children from the Republic of Congo, on the carbamylation of proteins upon the usage of cassava roots as a dietary staple. Upon ingestion of cyanogenic cassava, the linamarin is hydrolyzed to cyanide and undergoes albumin carbamylation. This mechanism triggers increased production of higher albumin carbamylation in
Impact of carbamylation on protein structures

Carbamylation can affect proteins structurally and functionally, with alterations in the interactions at protein-protein and intracellular levels leading toward the complete loss of function of the modified proteins. When a carbamoyl group modification happens on the amino acids of a protein, it inherently results in the neutralization of one positive charge, affecting its interaction with the cellular milieu environment. This may result in the destabilization of the protein at various levels of structure, accounting for severe conformational irregularities. As Stokes radii (also called hydrodynamic radius) of the proteins depends on the size of the protein as well as the conformation in solution, lysine carbamylation of bovine serum albumin has been shown to increase Stokes radii and decrease the stability against urea denaturation.

Instability in alpha-crystallin due to cyanate-induced carbamylation has been observed and well documented. Alpha-crystallin, on modification, undergoes structural changes leading to the thiol group exposure onto the surface, enabling oxidation and undesired disulfide bridges at intermolecular levels.

With certain physiologically relevant proteins, the exposure of specific functional groups due to structural changes induced by carbamylation can contribute towards autoimmune reactions (Rheumatoid arthritis) as in neoantigens (carbamylated albumin, carbamylated vimentin, etc.). Carbamylation of monomers, such as in type-I collagen, can result in an incorrect fiber assembly with shorter, denser structures. Carbamylation has been cited as the primary factor affecting collagen fibrillogenesis, reducing fiber resilience overall. Another such occurrence has been observed for actin and tubulin filaments, where carbamylation causes disorderliness in the structure and assembly of microtubules.

Protein carbamylation in aging and diseases

The average human lifespan is increasing worldwide due to advances in science and technology and improving patient care. On the flip side, this also has led to an increasing prevalence of age-related pathologies. Scarcity. However, our group has extensively researched the effect of carbamylation on Microtubule-associated protein Tau (MAPT) aggregation and the associated structural change. Carbamylation, being accounted for tau protein structure variation, has also been credited with its altered amyloidogenic properties.

In some cases, carbamylation of protein acts as a beneficiary condition; Haemoglobin (Hb) is transformed into carbamylated haemoglobin (cHb) in high urea concentrations. All the N-terminal valines present in the α and β-subunits of Hb are altered by the reaction making the protein lose its structural properties. The cHb contains more oxygen affinity than its non-carbamylated counterpart, and thus the urea treatment is given to patients suffering from sickle cell anemia.

Functional implications of carbamylation on proteins

Carbamylation affects the functionality of several proteins, hormones, and enzymes, leading to the complete loss of its activity in the biological system. Examples include glutamate dehydrogenase, aspartate aminotransferase, 6-phosphogluconate dehydrogenase, superoxide dismutase, etc. The dysfunction can be attributed to many factors, from carbamylation at specific amino acids affecting the protein, to the difference in the molecule's stereochemistry causing partial or complete inactivation.

Erythropoietin, on carbamylation, becomes devoid of its hematopoietic property but with no change in its cryoprotective abilities. Another such example is the carbamylation of tissue inhibitor of metalloproteinase-2 which completely removes its inhibitory properties present against matrix metalloproteinase-2 otherwise in its native state. There are other cases where a gain of function is witnessed with carbamylation, such as an anti-inflammatory native peptide exhibiting pro-inflammatory activities post-carbamylation in an anti-microbial peptide.
proteins results in the increased half-life of a protein in the cell. Both factors increase the load of carbamylate proteins in the cell, and that may cause a gain of toxic function in a protein. Figure 2 describes the impact of carbamylated proteins and their interaction in various age-related chronic diseases.

**Cataract**

Cataract is one such age-related disease that progressively opacifies eye lenses leading to disturbances in vision, eventually blindness. Senile cataract, amongst other types, is caused by altered lens protein – crystallin. The cells in the eye lens are enucleated, which means they are non-renewable and have a high shelf-life, so they undergo molecular deterioration over a longer time frame. Crystallin is one such protein present in the lens that is considered as a good study model for the molecular ageing of proteins. Specific subtypes of crystallins, namely αA-, βB2- and γS, were found to be carbamylated over time in the elderly population, which upon carbamylation reaction, results in crystallin aggregation and sometimes in the nucleation of native crystallin driving towards accumulation.

**Chronic Renal Failure**

Renal failure is considered a worldwide health concern mainly because of the contribution of age-related decline, the growing diabetic population, and lifestyle-related conditions such as obesity and hypertension. The end-remedy to chronic renal failure often requires transplantation or a lifelong dialysis procedure. Renal failure is known to have been caused by the progressive increase in serum urea levels, while its correlation with constitutive carbamylation reaction utilizes the cyanate-producing plasma isocyanic acid. Carbamylation in the myeloperoxidase pathway is also linked to renal failure and atherosclerosis. Carbamylation in renal failure has been reported to be associated with anemia and renal fibrosis, the decline in collagen-fibrinogen turnover, malnutrition, metabolic acidosis, free amino acid depletion, and chronic inflammation. The severity is proposed to vary based on the proteins' carbamylation degree.

**Atherosclerosis**

Carbamylated lipoproteins of the vascular walls are characteristic of elevated uremia. Atherosclerotic lesions consist of high-density lipoproteins with a significant increase in carbamylation levels, mainly formed from the myeloperoxidase pathway involving isocyanic acid production. Alterations in specific key lysine residues (also prone to oxidation) affect the binding affinity towards ApoE/B receptors. Carbamylated collagen in atherosclerosis causes higher reactive oxygen species development, leading to matrix remodeling and fragilization of arterial wall plates.

**Rheumatoid arthritis**

Rheumatoid arthritis is a chronic autoimmune disease characterized by severe inflammation and joint destruction that has recently been linked to carbamylation. Different carbamylated sites on the peptides and serum proteins are currently studied for their applicability as anti-carbamylated-protein antibodies against rheumatoid arthritis diagnosis and therapy. The most common hypothesis revolves around carbamylated proteins of the immune system promoting complement activation and thereby leading to inflammation. Further, this inflammation from carbamylation has also been linked to other allied cardiovascular complications in patients with rheumatoid arthritis.

**Carbamylation in neurodegeneration**

The impact of carbamylated proteins in renal failure, cataracts, and diabetes is well documented, but carbamylation remains a largely undiscovered link to neurodegenerative disorders. Neurologically vital proteins such as microtubule-associated protein tau, α-synuclein, TAR DNA-binding protein 43, etc.,
which are rich in Lysine residues, may undergo carbamylation. Most cases of protein aggregation in neurodegeneration are directly linked to ageing. Therefore, DPMs such as carbamylation can have a pronounced effect during ageing and have severe ramifications in regulating the properties of aggregation-prone proteins. Gallart-Palau et al. observed an overall increase in the levels of increase in brain ureido-DPMs in dementia brains, and suggested a defective immune response action. They observed that carbamylation of lysine in brain proteins (Homocitrullination), a spontaneous uDPM, clearly contributes to brain proteinopathy in the dementia subtype and elicits a neuroinflammatory response. The role of hypoxia in ageing and neuroinflammation is also well studied, which shows that these uDPMs play a crucial role.

The first study of carbamylated protein in neurodegeneration was performed in 1972 by a group of scientists at the Kansas medical center, demonstrating the toxic effect of cyanate accumulation in Rat brains. They injected sodium cyanate and carbamoyl phosphate in rats at various concentrations and found rapid disappearance of free cyanate in the blood due to protein binding. They found a dose-dependent decrease in learning ability when the rats were injected with different cyanate concentrations. The results also showed the retention of carbamylate proteins in animals even after the cyanate discontinuation. As memory and learning are directly related to specific brain parts, the study implicated the importance of neuronal and synaptic protein and its irreversible modification due to carbamylation.

The above study was taken as a biochemical model for neurodegeneration in our laboratory, and we have reported that carbamylation causes irreversible modification of microtubule binding protein Tau proteins and the derived peptides. The study was performed on full-length Tau and the core-aggregating peptide fragment - PHF6. The control peptide fragments that could not aggregate were significantly aggregated upon the treatment of potassium cyanate (KCNO). Also, full-length recombinant native tau protein that did not exhibit fibrillation without external aiding agents, upon carbamylation exhibited robust fibrillation with a sigmoidal aggregation pattern via ThT assay. The fibrils were visualized via transmission electron microscopy (TEM) and using MD simulation we further determined that lysine carbamylation can modulate the aggregation kinetics of amyloidogenic proteins and peptides and impact amyloid assembly architecture.

Exploring the same objective of carbamylation impacting aggregation kinetics and amyloid assembly formation, we extended our study on short flanking lysine-rich peptide fragments of tau protein. Many studies have depicted the effect of PTMs on the tau protein's microtubule-binding region (MBD) on Alzheimer's disease progression. Our study revealed major lysine-rich non-amyloidogenic hidden aggregation hotspots in unsuspected regions, that upon lysine carbamylation drive aggregation and formation of β-rich amyloid assembly. We also studied the cytotoxic effect of these carbamylated fibrils formed in SH-SY5Y neuroblastoma, which indeed was found toxic. Experimental observations were supported by Molecular Dynamic simulation that confirmed the strong intermolecular hydrogen bonding upon carbamylation as the key driving force leading to the aggregation of peptides.

In another unpublished study, we have shown that in α-synuclein, some of the imperfect KTKEGV repeat motifs could be an important driver of the aggregation upon carbamylation. In addition, we also observed that disease-specific mutation could simply be promoting aggregation by switching to a sequence that becomes amyloidogenic upon carbamylation.

Control and clearance of carbamylated proteins

Given the detrimental effect of various carbamylated proteins, especially in age-related disorders, various physiological clearance processes and different interference strategies have been extensively explored. Upon the involvement of carbamylated proteins load in disease progressions like Rheumatoid arthritis, cataract, and chronic kidney disease, the treatment targets the clearance of modified proteins via clearance pathways such as Ubiquitin proteasomal systems and Autophagy. Therapeutic approaches to increase the degradation of carbamylated proteins via degradation by proteosome and other physiological mechanisms can be employed. For free carbamylated amino acids, we can use the cyanate scavengers as the therapeutic approach. In case of chronic urea load in the body due to various kidney diseases, Dialysis should be the practical approach to reduce the urea load and thereby decrease the carbamylation of proteins.
Conclusion

Although ureido degenerative protein modifications such as carbamylation were discovered in the 1990s, when it comes to ageing and neurodegeneration, they have been extensively studied and investigated only in the last decade. Now there are multiple studies available with clear evidence of the adverse effect of carbamylation on protein properties and physiological functions. We have also studied the aggregation propensities of carbamylated proteins implicated in neurodegenerative diseases such as tau and α-synuclein and have observed that for both proteins, fully carbamylated protein is more amyloidogenic than the native form. However, the information related to the impact of site-specific carbamylations is limited to only peptide models, and methods to induce carbamylation in full-length protein at specific sites still need to be developed. Carbamylation-modified proteins are intriguing risk and prognostic factors for various age-related diseases and can also be explored as valuable biomarkers for disease diagnostics.

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Conflict of interest

All authors declare no conflict of interest.

References

11 Stark GR, Reactions of cyanate with functional groups of proteins. 3. Reactions with amino and carboxyl groups. Biochemistry, 4 (1965) 1030.


