FILK Freiberg Institute

Literature study on the use of glutaraldehyde for crosslinking biological tissues

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1. Objective of the study

The pursuit of safer use of chemicals and zero discharge of hazardous chemicals has resulted in an ongoing chemical assessment and regulatory activity. Glutaraldehyde as a pure chemical shows acute toxicity and is eye irritant as typical for aldehyde-type substances. It is sensitizing by inhalation and skin contact. Therefore, glutaraldehyde has been included in the SVHC Candidate List in 7/2021 as sensitizer.

The discussion about regulatory concerns and potential risks puts a lot of uncertainty on users of glutaraldehyde as cross-linking agent as well as on users of glutaraldehyde cross-linked products. The aim of the present study is therefore to provide an overview on research results of reactions of glutaraldehyde with proteins, potential reaction mechanisms and reaction products. The focus is set on biological tissues, namely collagen. The use of cross-linked collagen in the medical field is well monitored and subject to particularly tight regulations. The findings and conclusions for reaction mechanisms of glutaraldehyde with collagen in biological tissues in the medical field will be also applicable to other applications, e.g. tanning. Literature and own findings on residual free glutaraldehyde after cross-linking reactions are summarized. Conclusions will be drawn towards a safe use of glutaraldehyde as a cross-linker.

2. Reactions between glutaraldehyde and proteins

Glutaraldehyde (1,5-Pentanedial, C5H8O2) is a bifunctional aldehyde, which is established in different areas as a versatile cross-linking agent for various substrates. Thus, glutaraldehyde is used for the chemical stabilization or covalent coupling of proteins and enzymes (Richards and Knowles, 1968; Avrameas and Ternynck, 1969; Albarghouthi, 2000; Macquarrie and Bacheva, 2008) and in cross-linking of collagenous products (Nimni et al., 1987; Hayashi, 1994; Damink et al., 1995; Bigi et al., 2001; Fathima et al., 2004; Usha and Ramasami, 2005; Dong and Lv, 2016; Felician et al., 2018; Meyer, 2019). In the leather production process it is established as a metal free tanning agent (Fein et al., 1959; Filachione, Fein and Harris Jr, 1964; Heidemann, 1993; Covington, 2009). Due to its high reactivity, glutaraldehyde reacts fast with the substrate. The reaction mechanisms leading to the cross-linking of proteins are versatile and not yet completely clarified in detail.

2.1. Glutaraldehyde in aqueous solution

Glutaraldehyde in aqueous solution does not consist of a defined concentration of monomeric glutaraldehyde molecules. It contains a variety of monomeric, oligomeric or circular adducts, which derive from reactions of glutaraldehyde molecules among each other (Aso and Aito, 1962; Richards and Knowles, 1968; Hardy, Nicholls and Rydon, 1969; Korn, Feairheller and Filachoine, 1972; Whipple and Ruta, 1974; Peters and Richards, 1977; Ruijgrok, Boon and De Wijn, 1990; Tashima et al., 1991; Kawahara et al., 1992). Migneault et al. describe 13 possible species which can be detected depending on temperature, pH value, concentration and solvent type (see (Migneault *et al.*, 2004) und references therein). Figure 1 illustrates this variety with some selected structures. These glutaraldehyde adducts are reactive themselves and contribute to the cross-linking of proteins, resulting in a multitude of cross-links with different length and structure instead of a uniform cross-linking product.

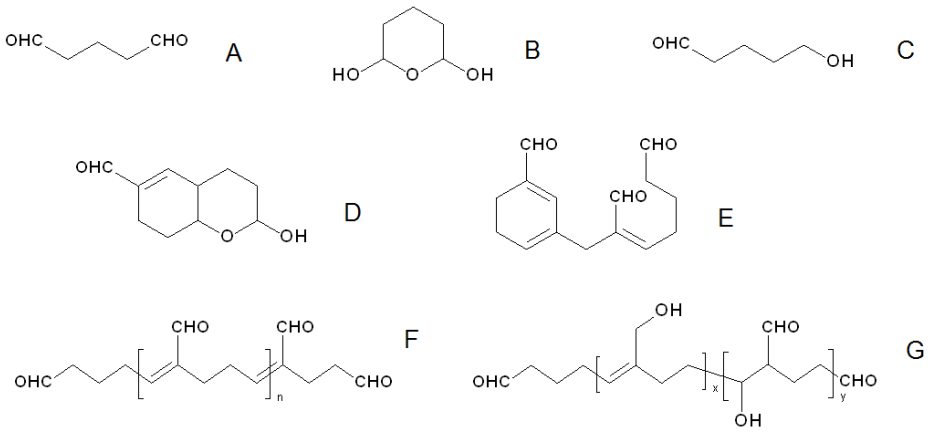

Figure 1: Selected structures of glutaraldehyde in solution: glutaraldehyde (A), monomeric (B, C), cyclic (B,D, E) and oligomeric (F, G) variants, appearing with increasing pH (A, B, C < F < D, E, G) (based on (Migneault et al., 2004)).

2.2. Crosslinking reactions

Among different bifunctional aldehydes, glutaraldehyde belongs to the most reactive ones (Fein et al., 1959; Bowes and Cater, 1968; Migneault et al., 2004). It reacts fast and easily with primary amino groups (e. g. α-amino groups of free amino acids or ε-amino groups of lysine or hydroxylysine side chains) and thiol groups (cysteine side chains, which is irrelevant for collagen), but only partially with the side chains of tyrosine and histidine (Habeeb and Hiramoto, 1968). The amino groups of lysine side chains are the preferred point of attack resulting in cross-links, if two protein moieties are involved (Habeeb and Hiramoto, 1968; Peters and Richards, 1977; Damink et al., 1995). Possible reaction mechanisms are discussed in the literature, which include the intermediate formation of Schiff bases by direct reaction between glutaraldehyde carbonyl groups and protein amino groups, as well as Michael addition type reactions via α,β-unsaturated aldehyde species. Instable intermediates are stabilized by further reactions or rearrangements with the formation of stabilizing conjugated double bonds. Furthermore, N-alkylated pyridinium compounds can be generated by cyclization, dehydration and isomerization (Hardy, Nicholls and Rydon, 1976; Monsan, Puzo and Mazarguil, 1976; Hardy, Hughes and Rydon, 1979; Lubig *et al.*, 1981; Damink *et al.*, 1995; Migneault *et al.*, 2004). Starting with an aqueous glutaraldehyde solution containing a multitude of glutaraldehyde adducts, the reaction between glutaraldehyde and proteins results not in a defined and uniform product but in a variety of products with oligomeric cross-links of different length with the glutaraldehyde concentration showing a great influence (Damink et al., 1995). Figure 2 shows some selected cross-links.

The reaction between glutaraldehyde and amino groups proceeds fast. Investigations of the reaction kinetics led to a pseudo first order reaction with a formation rate of glutaraldehyde-protein-linkers of ca. 1 s^{-1}·mol^{-1}·l (Hopwood, Allen and McCabe, 1970). The reaction rate increases with the glutaraldehyde concentration, pH value and temperature (Fein *et al.*, 1959;

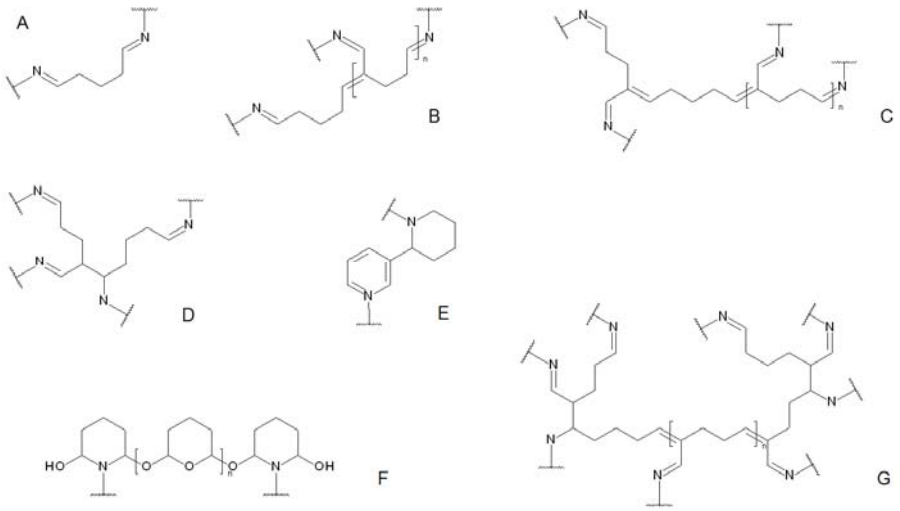

Figure 2: Selection of possible cross-links between glutaraldehyde and proteins: simplest possible cross-link (A), branched cross-links (B, C, D, G), linkers containing cyclic species (F) and pyridinium derivatives (E). Based on intermediate products from (Damink et al., 1995; Migneault et al., 2004) assuming complete reaction of all aldehyde groups.

Hopwood, Allen and McCabe, 1970). Studies on glutaraldehyde and primary amines showed that at neutral pH and 30 °C the reactions proceed fast in the first five minutes and the free amino groups are completely converted within ca. 30 minutes (Okuda *et al.*, 1991). Cross-linking of tissues can take more time. In the literature is reported that skin is tanned within 60 minutes (Damink *et al.*, 1995). In the case of more compact material like tendons, thorough washing of the cross-linked product is crucial in order to remove excess unreacted glutaraldehyde, which otherwise could be detected over several weeks (L. L. Huang-Lee, Cheung and Nimni, 1990). The stoichiometry is described as 3 - 4 glutaraldehyde molecules per amino group (Korn, Feairheller and Filachoine, 1972; Okuda et al., 1991). The reaction proceeds in a wide pH range (from 3 to 9) with the amount of reacted amino groups increasing with pH (Hopwood, Allen and McCabe, 1970; Monsan, Puzo and Mazarguil, 1976; Okuda et al., 1991). The optimum of cross-linking is achieved at pH values between 6 and 9 (Damink et al., 1995). The pH also influences the type of reaction products: polycondensates were observed primarily at pH values lower than 7 (Lubig et al., 1981). Generally, the cross-linking degree depends on incubation time, temperature and glutaraldehyde concentration (Ruijgrok, De Wijn and Boon, 1994a). It was observed on sheets of rat dermal collagen that a glutaraldehyde cross-linked product shows no cytotoxicity, when no free glutaraldehyde is detected (which can be achieved by washing and rinsing or removing residual glutaraldehyde with lysine), (Hey *et al.*, 1990).

The covalent bonds which are introduced by reaction of glutaraldehyde with amines are stable even under the extreme conditions of total acidic hydrolysis (Bowes and Cater, 1968). This becomes noticeable in the amino acid analysis, where the decrease of lysine and hydroxylysine content correlates with the cross-linking degree. The reversibility of reactions between glutaraldehyde and primary amines in solution is reported to be negligible (Hopwood, Allen and McCabe, 1970; Okuda *et al.*, 1991). It is further reported for glutaraldehyde cross-linked tendons that degradation and reversibility of cross-links cannot be completely excluded. However, potential cytotoxic effects of cross-linked tendons on fibroblasts are ascribed rather to free unreacted glutaraldehyde due to insufficient washing. Thoroughly rinsed cross-linked tendons show no cytotoxic effects (L. L. Huang-Lee, Cheung and Nimni, 1990).

3. Industrial use of Glutaraldehyde

3.1. Brief overview of aldehydes as chemicals in general

Aldehydes are very reactive compounds; the carbonyl group easily enters into additions, condensations, and polymerizations. Aldehydes are used in a broad variety of different applications. It ranges from synthesis, intermediates in the manufacture of plastics, solvents, dyes, pharmaceuticals, perfumes, and flavouring. Lower aliphatic aldehydes are used as raw materials for synthesis, for plastics and synthetic resins (aminoplasts, phenoplasts), as disinfectants, and for tanning. The higher aliphatic aldehydes and aromatic aldehydes are found in the production of fragrances (aldehyde notes in perfumes) and flavours, pharmaceuticals, pesticides and colourings. Many aldehydes occur in nature e.g. as flavouring agents. Among these are benzaldehyde (almond), cinnamaldehyde (cinnamon), citronellal, citral, and vanillin (flavouring agent of vanilla beans) (RÖMPP, 2018). Since aldehydes are reactive components, aldehydes occurring in food are also classified with regard to possible health risks. Table 1 shows selected examples. However, content levels in food are considered safe.

With respect to tonnage, only formaldehyde and glutaraldehyde are used to a significant amount as intermediates for plastic production and disinfection. Formaldehyde is manufactured in and / or imported to the European Economic Area, at ≥ 1.000.000 tonnes per annum (ECHA, 2021a). Glutaraldehyde is manufactured in and / or imported to the European Economic Area, at ≥ 10.000 to < 100.000 tonnes per annum (ECHA, 2021b).

Table 1: Selected aldehydes found in food. The listed health risk refers to the pure aldehyde. All listed aldehydes cause serious eye irritation as pure substances.

	Type of aldehyde	**Hazard**	**Concentration [ppm]**	**Literature**
Coffee	Hydroxymethyl-furfural	potentially mutagenic	605	(Kanjahn and Maier, 1997)
	Furfural	potentially carcinogenic	255	(Eseyin and Steele, 2015), (Macheiner *et al.*, 2021)
Instant coffee			3,000	(Kanjahn and Maier, 1997)
Cinnamon	Cinnamaldehyde	Risk of skin sensitization	25,800	(Lungarini, Aureli and Coni, 2008)
Orange juice			122	(Friedman, Kozukue and Harden, 2000)
Chocolate			980	(Lungarini, Aureli and Coni, 2008)
Lemon peel	Citral	Risk of skin sensitization	937	(Ahmad *et al.*, 2006)
Lemon grass			68,000	(Rana, Das and Blazqeuz, 2016)
Vanilla beans	Vanillin	Eye irritant	25,000	(Walton, Mayer and Narbad, 2003), (Gassenmeier, Riesen and Magyar, 2008)

Glutaraldehyde is used for industrial, laboratory, agricultural, and medical purposes, primarily for disinfecting and sterilization of surfaces and equipment (NCBI, 2021). Due to its reactivity, glutaraldehyde is a strong disinfectant. It is widely used in water treatment and high level disinfection, and in fogging and cleaning of poultry houses. Glutaraldehyde has a broad spectrum of antibacterial activity including antiviral and sporicidal activity and is therefore used to preserve and disinfect devices and instruments in the cosmetic industry and in medicine, which cannot be sterilized by heat. When strongly diluted (5 ppm), glutaraldehyde is effective against bacteria that cause corrosion (*Desulfovibrio desulfuricans*) and is therefore used in oil fields and fracking technologies as an anti-corrosion agent. Glutaraldehyde is formaldehyde free, biodegradable, non-carcinogenic, non-persistent and non-bio-accumulative.

Health care and water treatment are projected to be the dominant application segments of the glutaraldehyde market (*Glutaraldehyde Market Research Report: Market size, Industry outlook, Market Forecast, Demand Analysis,Market Share, Market Report 2021-2026*, no date). Around 45 % to 50 % of the glutaraldehyde solutions are used as a microbicide in evaporative recirculating cooling towers and large air-conditioning systems. (*Glutaraldehyde Market Insight and Trends 2027 - TMR*, 2021). Future market drivers are expected to be the health care industry, as well as water treatment application and pipeline projects.

3.2. Use of Glutaraldehyde as product component

Due to the reactivity of its two aldehyde groups, glutaraldehyde is an important starting material in chemical syntheses, e.g. of ring compounds and heterocycles. (RÖMPP, 2018).

The crosslinking ability of glutaraldehyde is based on its two terminal aldehyde groups, which easily react with amines and polyhydroxy compounds (e.g. cellulose derivatives, polyvinylalcohol and polyvinylacetates) to produce stable bonds. This crosslinking ability is used in many different applications.

Fixatives for biological tissue

In electron and light microscopy, preparation of thin sections of biological tissues for microscopy requires stabilization. Glutaraldehyde is used for preparation because of its reactivity and crosslinking ability towards proteins (Prentø, 1995). It is also used for embalming fluids and conservation.

Crosslinking in medical applications

Glutaraldehyde is used in medical applications for e.g. bioprosthetic valves (Southern *et al.*, 2000) in skin tissue engineering (Sundar *et al.*, 2021; Blackstone, Gallentine and Powell, 2021), and for collagen injections for incontinence treatment (Kumar, Benson and Bland, 2003). In bioprosthetic valves, glutaraldehyde cross-linked pericardium has been used for about 50 years. There have always been concerns due to the toxic potential of glutaraldehyde that is widely used in the treatment of pericardium, since any released residuals would be taken into the systemic circulation. In practice, there is no evidence of adverse clinical effects with commercial products. The toxicological profile for glutaraldehyde indicates the NOAEL (no observed adverse effects level) for chronic ingestion exposure to glutaraldehyde in rats is

4 mg/kg/day (Sundar *et al.*, 2021; Wilbur *et al.*, 2017), which is far higher than the levels expected to be released from biologic valves (Williams *et al.*, 2021; Naso *et al.*, 2013).

Enzyme immobilization

In biotechnology, glutaraldehyde is as a proven cross-linker. It is one of the most widely used reagents in the design of biocatalysts (Barbosa *et al.*, 2014; Betancor *et al.*, 2006; Walt and Agayn, 1994). The high reactivity of glutaraldehyde toward proteins at neutral pH is based on the presence of several reactive residues in proteins and molecular forms of glutaraldehyde in aqueous solution, leading to many different possible reaction mechanisms. Enzyme immobilization represents a good example to illustrate the use of glutaraldehyde as protein crosslinking reagent (Migneault et al., 2004). A fairly rigid control of reaction conditions is needed to achieve efficient insolubility of each different enzyme due to their structural variability. Nonetheless, the resulting enzyme derivatives generally show good stability and thus can be reused. Examples for industrial applications are given in Table 2. For one-enzyme reactions, it is also possible to immobilize whole cells without isolating the enzyme. Cells are cultivated under conditions under which they have the highest possible enzyme activities and, after suitable copolymerization (e.g. addition of a neutral protein and crosslinking with glutaraldehyde), the inactive cells are used directly as immobilized biocatalysts. In their natural microenvironment, enzymes often have higher levels of stability and activities than isolated enzymes (Kourist and Hartmann-Schreier, 2013).

Table 2: Examples for industrially used enzymes immobilized by glutaraldehyde. Data taken from (Kourist and Hartmann-Schreier, 2013)

Cell species	Enzymes	Application	Method for Immobilization
Actinoplanes missouriensis	Glucose-Isomerase	Isomerate sugar production	Gelatin cross-linked with glutaraldehyde
Bacillus coagulans			Glutaraldehyde crosslinking
Bacillus sp.	β-Galactosidase	Lactose-Hydrolysis	
Escherichia coli	Penicillin-Acylase	Production of 6-aminopenicillan acid from penicillin	Encapsulation in gelatine cross linked with polyacrylamide and glutaraldehyde
Mortierella vinacea	α-Galactosidase	Raffinose hydrolysis	Glutaraldehyde crosslinking
Proteus rettgeri	Penicillin-Acylase	Production of 6-aminopenicillan acid from penicillin	Glutaraldehyde bonding to glycidyl methacrylate
Streptomyces olivaceus	Glucose Isomerase	Isomerate sugar production	Bonded to ceramic support by crosslinking with polyamine and glutaraldehyde
Pseudomonas sp.	Cephalosporin-Amidase	Production of 7-aminocephalosporanic acid from cephalosporin	Adsorption to polystyrene-and crosslinking with glutaraldehyde

Cellulose crosslinking in wood, paper and textiles

Crosslinking with glutaraldehyde is used in the wood, paper and textile industry to functionalize cellulosic or cotton materials. It is used as water repellent treatment for wood (Xiao *et al.*, 2010) in order to minimize swelling and as wood bonding (Xi *et al.*, 2021). In the paper industry, it improves the wet strength (Xu, Yang and Deng, 2002). The treatment of textiles with glutaraldehyde aims at increasing the crease resistance (Dehabadi, Buschmann and Gutmann, 2013)

The STANDARD 100 by OEKO-TEX® limits glutaraldehyde with a value of 1000 mg/kg for all product classes.

Tanning agent

Tanning names a technical process that stabilizes the fiber structure of an animal skin and prevents it from decay and decomposition. Chrome tanning and glutaraldehyde tanning are the most important tanning processes worldwide. Glutaraldehyde reacts with the free amino groups present in the collagen fibrils of the hides. In addition to glutaraldehyde crosslinking, a variety of different processes turns a skin into leather. Glutaraldehyde tanning is used for ca. 15 % of the world leather production. Approximately 25 %–50 % of the glutaraldehyde solutions (world production) are used in the leather industry (*Glutaraldehyde Market Insight and Trends 2027 - TMR*, 2021).

Glutaraldehyde is mainly used in the first tanning step, which is followed by post-tanning procedures, fatliquoring, dyeing, and functionalization. The treatment includes additional steps for pH adjustment and washing steps in order to remove unbound chemicals. The amount of free glutaraldehyde in leathers therefore depends on the number and kind of washing steps in the leather production process. As the textile standard by OEKOTEX, also the LEATHER STANDARD 100 by OEKO-TEX® limits glutaraldehyde with a limit value of 1000 mg/kg for all product classes.

At FILK in 2021, the glutaraldehyde contents of commercial leathers were surveyed over a period of six months. Approximately 10 % of the 600 leather samples tested according to DIN EN ISO 17226-1 contained free glutaraldehyde, of which more than half were below the quantification limit of 2 mg/kg. All measured values for free glutaraldehyde were below 1000 mg/kg. Only one of the samples contained more than 100 mg/kg free glutaraldehyde.

Cosmetics

Glutaraldehyde is used as preservative in cosmetic applications. It is mentioned in the EC Regulation 1223/2009 on cosmetics, Annex V (EUR-LEX, 2021) and is restricted to 1000 mg/kg. Labelling is required if the glutaraldehyde concentration in the finished product exceeds 0.05 %, i.e. 500 mg/kg.

4. Usage of glutaraldehyde for the cross-linking of biological materials

Glutaraldehyde is an important reagent in the biomedical field. Among the numerous chemical cross-linkers that are utilized to improve mechanical properties and stability of biomaterials, glutaraldehyde is one of the most frequently used (Schmidt and Baier, 2000; Reddy, Reddy and Jiang, 2015). Treatment with this agent leads to a marked increase in thermal stability and significantly reduces tissue antigenicity as well as biodegradation (Nimni *et al.*, 1987b; Beauchamp *et al.*, 1992; Ruijgrok, De Wijn and Boon, 1994b; Meyer, 2019). Since its introduction into biomedicine in the late 1960s (Carpentier *et al.*, 1969), glutaraldehyde has been applied extensively as a cross-linking agent for the preparation of collagen-based xenogeneic tissues in a variety of human clinical applications. Such applications include the fabrication of heart valves (Duran, Gallo and Kumar, 1995; Corno *et al.*, 2001; Thomson *et al.*, 2001), vascular prostheses (Sawyer *et al.*, 1987), skin substitutes (Schechter *et al.*, 1975; Griffiths and Shakespeare, 1982), materials for guided tissue reconstruction (Quteish and Dolby, 1992; Bennett *et al.*, 1995) and drug delivery matrices (Jayakrishnan and Jameela, 1996). Numerous studies have shown successful and safe clinical usage of glutaraldehyde cross-linked devices (Dardik *et al.*, 2002); (Eichinger *et al.*, 2002; Chao and Torchiana, 2003; Ozaki *et al.*, 2014). Therefore, today this cross-linking procedure is generally accepted for medical applications (McPherson, Sawamura and Armstrong, 1986; Jayakrishnan and Jameela, 1996).

4.1. Review of the cytotoxicity of glutaraldehyde

During the last years, the use of glutaraldehyde to stabilize biological materials for medical applications has been intensively discussed. Although treatment with glutaraldehyde results in good improvement in mechanical properties and also suppresses immunological recognition of the cross-linked material (Nimni *et al.*, 1987), it does not completely eliminate the immune response of the body (Schmidt and Baier, 2000). Residual glutaraldehyde as well as unstable glutaraldehyde polymers have been discussed to be liberated from cross-linked devices upon implantation and biodegradation and thereby elicit cytotoxicity. Speer et al. showed that there is extractable glutaraldehyde present in cross-linked collagen materials and that a continuous release of residual aldehyde can be monitored (Speer *et al.*, 1980). In another study, the cytotoxicity of glutaraldehyde cross-linked dermal sheep collagen was tested using skin fibroblast over a culture period of 42 days (van Luyn *et al.*, 1992). It was demonstrated that a bulk of cytotoxic products were released after 6 days and mainly comprised residual cross-linking agent. However, a low secondary cytotoxicity was measured over the whole culture period of 42 days. This release of cytotoxic products was attributed to enzymatic degradation of the cross-linked implant, whereby unreacted glutaraldehyde entrapped in the collagen matrix is released. Toxic effects of the cross-linking procedure using glutaraldehyde can be reduced or even eliminated by thoroughly rinsing the implant before use (Speer *et al.*, 1980; L. L. H. Huang-Lee, Cheung and Nimni, 1990) or by post-treatment of the materials with amino acids, e. g. glycine or glutamic acid (Gough, Scotchford and Downes, 2002; Park *et al.*, 2017).

Regarding the cytotoxicity of glutaraldehyde, it is noteworthy that the initial concentration used for the cross-linking of biological materials has a significant effect on their biocompatibility, both, *in vitro* and *in vivo*. It has been shown that the amount of residual glutaraldehyde molecules diffusing out of cross-linked devices is dependent on the concentration used for cross-linking (McPherson, Sawamura and Armstrong, 1986). Studies

employing higher concentration of glutaraldehyde generally observed inflammation and cytotoxicity, whereas in studies using glutaraldehyde concentration below a certain level an excellent biological performance of the cross-linked materials has been found (Jayakrishnan and Jameela, 1996). This dependency might be due to the mechanism of the cross-linking reaction. Cheung et al. demonstrated that in the case of reconstituted collagen fibres stable intramolecular cross-links were formed throughout the fibres at low concentration of glutaraldehyde (Cheung *et al.*, 1985). At high concentration, a rather fast generation of an intermolecular network of crosslinks was observed. This network consisted of high molecular weight glutaraldehyde polymers creating an artificial chemical barrier on the surface of the collagen fibres and preventing further glutaraldehyde molecules from penetrating through this barrier without becoming incorporated into the polymers. Consequently, with higher concentration of glutaraldehyde, more residual cross-linking agent is present in the cross-linked material increasing the possibility of cytotoxic effects.

Several studies have examined the critical level of glutaraldehyde to be used for cross-linking biological materials. However, the results obtained are not consistent and vary in terms of the cross-linking procedure as well as the test applied for assessing cytotoxicity. McPherson et al. and Meyer et al. found a glutaraldehyde concentration below 0.1 % to be non-cytotoxic (McPherson, Sawamura and Armstrong, 1986; Meyer, Baltzer and Schwikal, 2010), whereas Sun et al. and Lai et al. could use 0.3 % without observing toxic effects in their studies (Sun, Feigal and Messer, 1990; Lai, 2014). Own unpublished results showed a non-toxic behaviour of collagen-based membranes cross-linked with glutaraldehyde solutions with a concentration of up to 10 % towards skin fibroblasts in direct and indirect contact (Figure 3). Furthermore, a decellularized bovine pericardium already in use for clinical application and cross-linked with 8 % glutaraldehyde was shown to have no cytotoxic implication (Umashankar, Kumari and Mohanan, 2012; Reddy, Reddy and Jiang, 2015). During surgical operations of aortic aneurysm, even a 25 % glutaraldehyde solution was directly applied into the patients in order to strengthen the distal aorta and the authors report no adverse events (Rinaldi *et al.*, 1995).

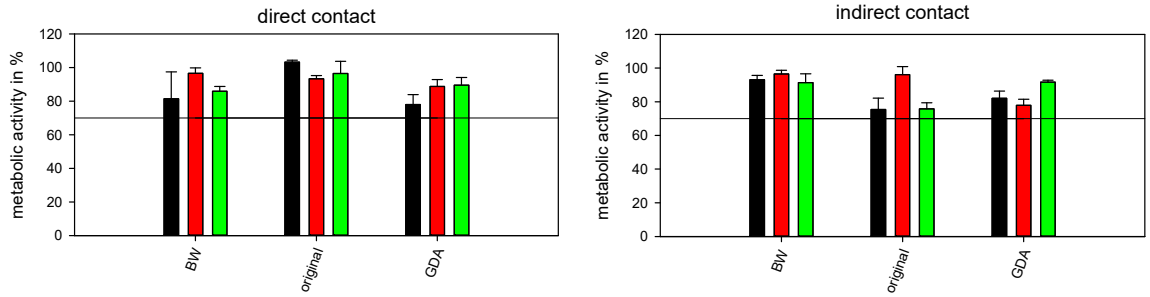

Figure 3: Metabolic activity of murine fibroblasts after direct (left) or indirect (right) contact with collagen membranes non-cross-linked (original) or cross-linked with glutaraldehyde (GDA). BW displays a control sample. Substances that limit the metabolic activity of the cells to values below 70 % (black line) are classified as cytotoxic.

All above mentioned results apply to glutaraldehyde cross-linked materials that are implanted in the human body, used for dressing open wounds or taken up orally. Such materials are already on the market and certified according to the European medical device regulations.

If the materials have only contact to intact skin, possible cytotoxic effects of glutaraldehyde are negligible. Studies of the *in vitro* penetration of glutaraldehyde in samples of skin from

rats, mice, rabbits, guinea pigs and humans showed, that less than 1 % of the applied cross-linking agent penetrated the skin (Beauchamp *et al.*, 1992). In this case, the presence of residual glutaraldehyde may even be of advantage because of its biocidal effects (Speer *et al.*, 1980).

4.2. Metabolism of glutaraldehyde cross-linked materials in vivo

Just like the chemical toxicity of glutaraldehyde, the metabolic mechanism by which this chemical is degraded *in vivo* still is a matter of debate and remains relatively unknown so far. Studies using radiolabelled glutaraldehyde showed that the majority of radioactivity is transformed both, *in vitro* and *in vivo*, to carbon dioxide (Beauchamp *et al.*, 1992). In experiments with rats, glutaraldehyde was rapidly converted to non-reactive metabolites by the liver and finally eliminated by the kidney (Ranly and Horn, 1990). However, these authors did not find a significant conversion of glutaraldehyde to carbon dioxide. Shi et al. used the mammalian liver post-mitochondrial fraction (S9) to study the metabolism of residual glutaraldehyde in animal-derived biomaterials (Shi *et al.*, 2020). The S9 system comprises a variety of ubiquitously distributed enzymes and plays an essential role in drug metabolism. The main metabolic factor of S9 are monooxygenases from the cytochrome P450 family that are responsible for the oxidation and degradation of drugs and environmental chemicals. In the presence of the S9 metabolic system, glutaraldehyde was oxidized via the cytochrome P450-NADPH pathway resulting in a decreased toxicity of the cross-linked biomaterial. Glutaric acid was formed as the main metabolite during the catalytic process. A possible scheme for the metabolism of glutaraldehyde *in vivo* is presented in Figure 4.

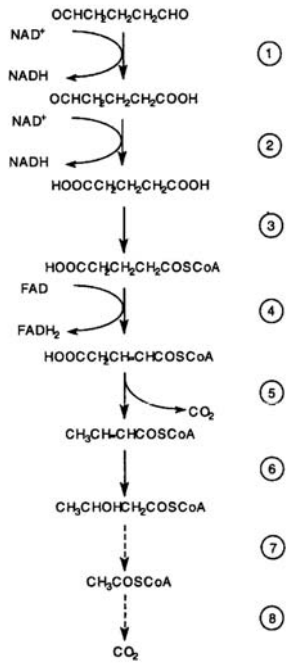

Figure 4: Postulated metabolism scheme for glutaraldehyde. (1) Oxidation of glutaraldehyde to glutaric γ-semialdehyde and (2) further oxidation to glutaric acid; (3) synthesis of glutaryl Coenzyme A; (4) oxidation to glutaconyl Coenzyme A; (5) decarboxylation to give crotonyl Coenzyme A; (6) hydration to β-hydroxybutyryl Coenzym A; (7) conversion to acetyl Coenzyme A; (8) oxidation to carbon dioxide (adapted from (Beauchamp et al.*, 1992))*

5. Summary

Glutaraldehyde is a widely used chemical in different industries. Glutaraldehyde is toxic, but biodegradable, non-carcinogenic, non-persistent and non-bio-accumulative. It is mainly applied as disinfectant and as crosslinking agent. As crosslinking agent, glutaraldehyde is used in industrial and medical applications. Glutaraldehyde reaction products are not toxic, but safe use requires proper application conditions to avoid unreacted traces and instable glutaraldehyde cross-linked polymers in the treated tissues. Liberation of free glutaraldehyde is attributed to the degradation of polymeric glutaraldehyde species rather than to a cleavage of the bond between the aldehyde and the amino groups of proteins. Accordingly, liberation of free aldehyde in medical applications could be prevented by readily available amino groups of amino acids.

The same applies for any other application, where glutaraldehyde is used as cross-linker (e.g. enzyme immobilization, tanning). Due to multiple washing steps and proper management of chemicals, the content of free glutaraldehyde in leathers was found to be below 1000 mg/kg in all 600 samples, that had been tested in 2021, which is in accordance with regulations of the EU e.g. on cosmetics and industry standards as STANDARD 100 by OEKO-TEX®.

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