Biocompatibility of choline salts as crosslinking agents for collagen based biomaterials†

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A series of novel choline based salts, some of which can be described as ionic liquids, are prepared and evaluated for their biocompatibility; when combined with collagenous biomaterials they exhibit good cell viability and adhesion properties as required for biomedical implant applications.

Tissue engineering is now gaining importance as an alternative to tissue or organ transplantation. With this technology, tissue loss or organ failure can be treated either by implantation of an engineered biological substitute or, alternatively, by *ex vivo* perfusion methods. The tissue-engineered products may be fully operational at the time of treatment or have the potential to integrate and form the expected functional tissue upon implantation.[1,2] In many cases, biomaterials are modified to repair or replace the damaged tissue. For example, progress has been made with engineered cardiovascular tissues such as heart valves[3,4] and blood vessels.[5,6] The development of biomaterials poses significant challenges since the implanted tissue is greatly influenced by the composition, architecture, three-dimensional environment of the implanted "scaffold" and the biocompatibility of the material. The material porosity and pore size distribution and continuity greatly influence the attachment of specific cell types and interaction of the biomaterials with the host. The inflammatory response to biomaterials has also been a major safety problem and many of the currently utilized biomaterials elicit inflammatory responses upon implantation.[7] Hence there is a need for stable biocompatible materials that are bio-acceptable and that, preferably, having induced new tissue growth, will degrade *via* normal biochemical pathways.

Biomaterials based on natural collagen are emerging as a potential source of such biocompatibility. Collagen is a protein mainly existing in human skin, skeleton, cartilage, tendons, ligaments and blood vessels, and it is a very important structural protein which supports and protects the organs and body. Collagen makes up 25–33% of total proteins in the body and 80% of the skin composition which is almost equal to 6% of bodyweight. It can be harvested from a number of sources including animals and processed by dissolution and re-precipitation, and hence is readily available as a biomaterial. It has appeared in a number of applications, for example collagen based wound dressings are advantageous over other systems because of unique characteristics such as weak antigenicity,[8] biodegradability,[9] and superior biocompatibility.[10] Collagen materials for long-term biomedical implant applications need to be crosslinked to improve the mechanical stability of the biopolymer and induce controlled biodegradability because, when not crosslinked, collagen membranes exhibit poor mechanical properties and rapid degradation limits their application as a biomaterial.[11] Glutaraldehyde (GA) is the most widely used chemical crosslinking agent[11] because it stabilizes collagen efficiently. The crosslinking is thought to involve the formation of Schiff bases.[12] However, GA-crosslinked biomaterials are poorly biocompatible with some cell lines including human fibroblasts, osteoblasts, Chang cells, and endothelial cells.[13–15] The side effects of GA treatment were attributed to the degradation of the GA-derived crosslinks and the continuous release of aldehydes contributing to *prolonged* toxic effects.[11,16] Several alternative crosslinking agents such as diphenylphosphoryl azide (DPPA) have been developed to minimize the toxicity.[17] Crosslinking with DPPA reduces degradation of collagen biomaterials and produces a more biocompatible material than crosslinking with GA.[11,18] However, there is a need to develop crosslinkers which are completely biocompatible and produce no inflammatory response for long-term implant applications. In this study choline salts have been used as crosslinkers with collagen based biomaterials and the biocompatibility of the resultant materials tested.

In recent years, ionic liquids (ILs) have been extensively investigated for use as replacement solvents for many chemical and biochemical reactions.[19–21] It has been shown that enzymes can be used to carry out bio-transformations in ILs.[22,23] A number of biocompatible hydrated ionic liquids have been identified, for example choline dihydrogenphosphate (CDP) with 20% dissolved water, and shown to be good solvents for proteins.[24,25] In the ionic solution some proteins are tremendously stabilized as compared to aqueous solutions,[26,27] thus leading to a variety of applications in drug delivery and sensor development. The presence of a solute amount of water in these systems is probably an important factor in supporting solubility and stability.

In the present study we have synthesized a number of novel choline salts, based on other anions from the "Generally Recognized as Safe" list or known Active Pharmaceutical Ingredients (Fig. 1), including choline lactate (CL), choline levulinate (CLu), and choline tartarate (CT). They have then been used as replacements for the most commonly used

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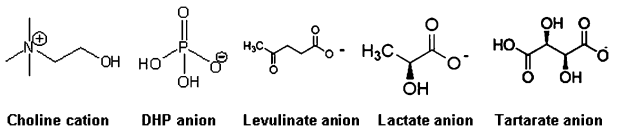

Fig. 1 Chemical structures of cation and anions.

chemical crosslinkers for collagen, such as GA, to reduce the toxicity of collagen based biomaterials.

Choline hydroxide (45% in methanol), L(+) lactic acid (98%), levulinic acid (98%), D(−) tartaric acid (99%) and bovine achilles tendon were purchased from Aldrich while phosphoric acid (85%) aqueous solution was purchased from Merck. The general synthesis involves (1 : 1 mole ratio) neutralization reaction of choline hydroxide and the corresponding acids (such as lactic acid, phosphoric acid, *etc.*) and dehydrating the reaction mixture at high temperature (70 °C) and reduced pressure to obtain the pure salt. For example, choline lactate (CL) (10 g) was made by a slow addition of lactic acid (4.66 g) to 45% methanolic choline hydroxide solution (13.93 g) in an ice bath and stirring it for about 2 hours at room temperature. Then the reaction mixture was evaporated at reduced pressures to obtain crude CL. To the crude compound, activated charcoal (approx. 1 g) was added, stirred with water and filtered. The filtrate was again evaporated to obtain the pure liquid in 98% yield. The other choline derivatives were prepared in a similar manner. All were characterized by electrospray mass spectroscopy and only the expected anion and cation were observed in each case. Electrospray mass spectroscopy analysis (cone ±35 V) : CL, *m*/*z* (relative intensity, %): ES^+, 103.7 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 89.8 (lactate, 100); CDP, ES^+, 103.7 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 96.7 (dihydrogenphosphate, 100); CLu, ES^+, 103.7 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 114.9 (levulinate, 100); CT, ES^+, 103.7 ($Me_3N^+CH_2CH_2OH$, 100); ES^-, 149.3 (tartarate, 100). CL and CLu are liquids at room temperature. CDP and CT are solids with melting points 185 °C and 150 °C, respectively. The latter two easily become liquid on addition of small amounts of water. In both cases 20% (w/w) is sufficient to produce a clear, fluid liquid at room temperature.

The collagen was isolated from bovine achilles tendon as an aqueous solution as per the literature procedure.[28] The crosslinking procedure typically involves the mixing of 5 g collagen solution (2 mg ml^{-1}) with 7.5 µl of an 8 mM aqueous solution of the crosslinker at room temperature. The mixture was then cast on a Teflon sheet and dried at room temperature overnight to produce a thin film. GA is well known to crosslink the collagen fibrils, *via* Schiff base formation, rendering the material water insoluble, as observed in this work with the ionic crosslinkers. In a certain respect this replicates the properties of the natural material, which *in vivo* must be insoluble. Water uptake tests show that the dry materials take up water to the extent of about 70–200% w/w after 24 hours, depending on the ionic liquid involved. There was little further uptake and after 6 months continuous immersion in a relatively large volume (20 ml) of pure water the samples remain intact. Since the original collagen is water soluble, this indicates that leaching of the ionic liquid from the material is not a significant process. IR and circular dichroism spectroscopy data (ESI†, Table S1 and Fig. S1) indicate that the triple helical structure of the collagen strand is retained in the crosslinked materials. Thermogravimetric analysis (ESI†, Fig. S2) shows that the materials are more thermally stable than the GA crosslinked materials.

The crosslinking involved here is probably hydrogen bonding based since the anions all share a feature of having multiple hydrogen bonding sites, both donor and acceptor. Natural collagen in the body is held insoluble by the action of such hydrogen bonds, between glycine and hydroxyproline units in particular. These units are disrupted by the acid treatment that creates soluble collagen. Hence our hypothesis is that the role of the crosslinker here is to provide an alternate means of forming these strong hydrogen bonds. The ionic nature of the crosslinker probably further strengthens the intermolecular interactions. The IR data (ESI†, Table S1) support this hypothesis. As described by Usha[29] *et al.* the collagen amide A band shifts to higher frequencies as a result of crosslinking, the Cr(III) ion producing the strongest effect. The data in ESI† Table S1 show that the shifts observed in the present materials are in some cases almost as substantial as Cr(III). This shift may stem from coordinate or H-bonding interactions of the amide nitrogen, hence in the present case where all of the anions are capable of multiple H-bonds, it seems likely that this is the origin of the crosslinking effect.

The choline based salts and the crosslinked collagen films were characterized for biocompatibility using the 3-[4,5-dimethyltriazol-2-y1]-2,5-diphenyltetrazolium (MTT) assay with cardiac fibroblast cells, as described in the literature[30] and the results were compared with control samples. The controls used were the neat medium (without collagen or crosslinker) and collagen crosslinked with GA. Further details of the MTT assay and method are provided in the ESI.† The results show (Fig. 2 and 3) the ability of the salts to support cell viability and proliferation. All the samples exhibited comparable biocompatibility to GA. In addition, CLu and CT showed better biocompatibility over GA in this assay.

Microscopic observation of rat fibroblastoma cells (L-929) confirmed that the cells in all cases were well grown and attached to the surface, although the CLu and CT crosslinked materials produce better growth compared to the sample crosslinked with GA. Cell spreading and attachment was monitored by fluorescence microscopy of fixed and fluorescently labelled cells (details provided in ESI†) and typical micrographs of the control (GA crosslinked) and CT crosslinked films are shown in Fig. 4. The results show that the cells were

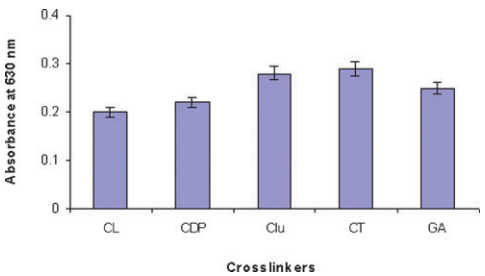

Fig. 2 MTT assay of choline based salts as compared to controls.

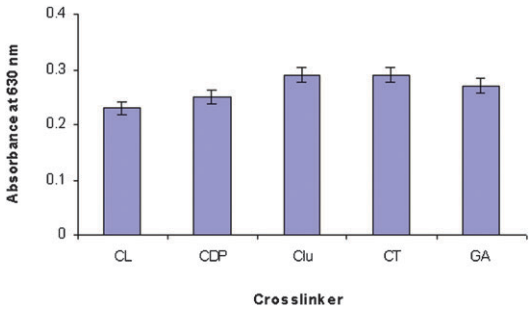

Fig. 3 MTT assay of the choline salt crosslinked biomaterials.

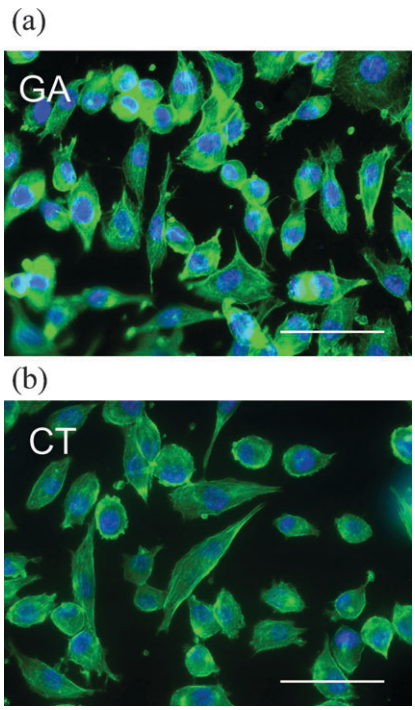

Fig. 4 Fluorescence micrographs of L-929 cells growing on GA and CT crosslinked materials. Actin cytoskeleton is stained with phalloidin (green), and the nuclear stain DAPI shows the cell nuclei (blue). Scale bars represent 50 μm.

found to be healthy, capable of proliferation, forming a good coverage on the material and attached very well to the surface.

Overall these results show that these choline based salts, in particular those based on the levulinate and tartarate anions, can be useful for biomedical implant applications. Studies pertaining to the mechanical properties of the salt crosslinked biomaterials will be reported separately and the *in vivo* studies of the biomaterial films for wound healing applications are underway.

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Notes and references

1 W. Yongzhong, J. B. Dominick, K. Hyeon-Joo, S. K. Hyun and L. K. David, *Biomaterials*, 2006, **27**, 4434.
2 C. S. Cho, S. J. Seo, I. K. Park, S. H. Kim, T. H. Kim, T. Hoshiba, I. Harada and T. Akaike, *Biomaterials*, 2006, **27**, 576.
3 T. C. Flanagan and A. S. Pandit, *Eur. Cells Mater.*, 2003, **6**, 28.
4 L. Xue and H. P. Greisler, *J. Vasc. Surg.*, 2003, **37**, 472.
5 L. E. Niklason, J. Gao, W. M. Abbott, K. Hirschi, S. Houser, R. Marini and R. Langer, *Science*, 1999, **284**, 489.
6 P. J. Schaner, N. D. Martin, T. N. Tulenko, I. M. Shapiro, N. A. Tarola, R. F. Leichter, R. A. Carabasi and P. J. DiMuzio, *J. Vasc. Surg.*, 2004, **40**, 146.
7 J. M. Anderson, *Trans. Am. Soc. Artif. Intern. Organs*, 1988, **34**, 101.
8 F. L. Nicolas and C. H. Gagnieu, *Biomaterials*, 1997, **18**, 815.
9 G. Goissis, E. Marcantoniojunior, R. A. C. Marcantonio, R. C. C. Lia, D. C. J. Cancian and W. M. de Carvalho, *Biomaterials*, 1999, **20**, 27.
10 S. Eun Song, K. Yeon, C. Taehoon, B. Hyun-Jung and L. Young Moo, *Biomaterials*, 2006, **27**, 2951.
11 E. Jorge-Herrero, P. Fernandez, J. Turnay, N. Olmo, P. Calero, R. Garcia, L. Freile and J. J. Castillo-Olivares, *Biomaterials*, 1999, **20**, 539.
12 L. H. H. Olde Damink, M. Dijkstra, M. J. A. Van Luyn, P. B. Van Wachem, P. Nieuwenhuis and J. Feijen, *J. Mater. Sci.: Mater. Med.*, 1995, **6**, 460.
13 L. L. H. Huang-Lee, D. T. Cheung and M. Nimni, *J. Biomed. Mater. Res.*, 1990, **24**, 1185.
14 M. J. A. Van Luyn, P. B. Van Wachen, L. H. H. Olde Damink, P. J. Dijkstra, J. Feijen and P. Nieuwenhuis, *Biomaterials*, 1992, **13**, 1017.
15 J. E. Gough, C. A. Scotchford and S. Downes, *J. Biomed. Mater. Res.*, 2002, **61**(1), 121.
16 C. E. Schmidt and J. M. Baier, *Biomaterials*, 2000, **21**, 2215.
17 H. Petite, V. Frei, A. Huc and D. Herbage, *J. Biomed. Mater. Res.*, 1994, **28**, 150.
18 S. Roche, M. C. Ronzie're, D. Herbage and A. M. Freyria, *Biomaterials*, 2001, **22**, 9.
19 J. S. Wilkes, *Green Chem.*, 2002, **4**, 73.
20 J. Golding, S. Forsyth, D. R. MacFarlane, M. Forsyth and G. B. Deacon, *Green Chem.*, 2002, **4**, 223.
21 P. Wasserscheid and T. Welton, *Ionic Liquids in Synthesis*, Wiley, Weinheim, 2003.
22 U. Krugle, M. Eckstein and N. Kaftzik, *Biocatalytic reactions in ionic liquids*, Wiley, Weinheim, 2003, p. 336.
23 J. L. Kaar, *J. Am. Chem. Soc.*, 2003, **125**, 4125.
24 K. Fujita, D. R. MacFalane and M. Forsyth, *Chem. Commun.*, 2005, 4804–3043; O. Winther-Jensen, R. Vijayaraghavan, J. Z. Sun, B. Winther-Jensen and D. R. MacFarlane, *Chem. Commun.*, 2009, 3041–3043.
25 K. Fujita, M. Forsyth, D. R. MacFarlane, R. W. Reid and G. D. Elliott, *Biotechnol. Bioeng.*, 2006, **94**, 1209–1213; K. Fujita, D. R. MacFalane, M. Forsyth, M. Y. Fujita, K. Murata, N. Nakamura and H. Ohno, *Biomacromolecules*, 2007, **8**(7), 2080–1213.
26 N. Byrne and C. A. Angell, *J. Mol. Biol.*, 2008, **378**, 707.
27 N. Byrne, L. M. Wang, J. P. Belieres and C. A. Angell, *Chem. Commun.*, 2007, 2714.
28 Z. Deyl and M. Adam, *The methodology of connective tissue research*, Joynson-Bruvvers, Oxford, 1st edn, 1976, p. 1.
29 R. Usha, A. Dhathathreyan, A. B. Mandal and T. Ramasami, *J. Polym. Sci., Part B: Polym. Phys.*, 2004, **42**, 3859–3865.
30 T. J. Mossmann, *J. Immunol. Methods*, 1983, **65**(1–2), 55.

