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Comparative Study of the Physical, Topographical and Biological Properties of Electrospinning PCL, PLLA, their Blend and Copolymer Scaffolds

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Abstract. Biodegradable polymers (blends, copolymers) could be the ideal materials for manufacturing of scaffolds for small diameter vascular graft. Such material characteristics as mechanical properties, chemical structure, nano- and micro topography, surface charge, porosity, wettability etc. are becoming the most important aspects for effectiveness of prosthesis biofunctionalization because of their great impact on cell adhesion, spreading, cell proliferation, differentiation and cell function. The aim of the study is to compare physical, topographical and biological properties of polycaprolactone (PCL), poly-L-lactic acid (PLLA), polycaprolactone + poly-L-lactic acid blend (PCL PLLA), L-lactide/Caprolactone copolymer (PLC7015) scaffolds fabricated with the same fiber thickness using electrospun technology. PCL PLLA scaffolds had the highest average pore area ($p<0.01$) and the lowest strength ($p<0.01$). PLC7015 scaffolds had the significantly lower average pore area ($p=0.03$) but the highest elastic deformation ($p<0.01$). Biological testing with MMSC (multipotent mesenchyme stem cells) demonstrated that after 72 hours of co-cultivation only on PCL and PLLA scaffolds cells entered to the active phase of adhesion process. We propose that physical and topographical properties of PCL, PLLA, their blend and copolymer are of a great dependence of chemical structure but could be changed during the manufacturing process that will lead to changes in biological properties.

1. Introduction

Autologous veins and arteries remain the only option for coronary artery bypass surgery and the vessels of choice for peripheral small diameter applications. When suitable natural vessels are unavailable for peripheral grafting, durable synthetic prostheses (ePTFE and Dacron) with poor long-term patencies are the only current alternative [1]. There is thus a large drive towards the development of tissue engineering and regenerative medicine (TERM) approaches to vascular grafts in order to not only provide suitable short-term solutions, but also to generate living neo-arteries that function as well as native vessels [2].

We propose that biodegradable polymers (blends, copolymers) could be the ideal materials for manufacturing of scaffolds for such small diameter vascular graft, as they have some advantages that

could be useful for these purposes. For instance polymers have more altitudinal synthetic flexibility with a variety of functional groups; multitudinous functional polymers show excellent mechanical and chemical robustness; polymers are easily patterned with state-of- the-art nano- and microtechnologies; many polymers are nontoxic and scalable, which are critical for real applications [3].

Thanks to the advantages of electrospun nanofibrous scaffolds, such as potential for mimicking of the extracellular matrix, nontoxicity, biocompatibility, biodegradability, permeability and low cost, PCL (poly(ε-caprolactone)) and PLLA (poly-L-lactic acid) are very promising for vessel graft development [4][5]. PCL contains significant number of alkyl units between the ester links, has more flexible polymer chain, low crystallization temperature, rubber-like behavior and a very long biodegradation period. PLLA contains more complex ester units in the chain, so it is more susceptible to degradation by hydrolysis and shows faster biodegradability but its applications are limited because of its lack of toughness. PLLA is rigid in a room temperature and at body temperature. A blend, or copolymer of PCL and PLLA, could therefore theoretically combine the properties of both polymers and provide resistant but ductile mechanical behavior, as well as prolonged biodegradability [6].

The cell-polymer interactions become the key questions of implanted prosthesis functioning and its biocompatibility. Such material characteristics as mechanical properties, chemical structure, nano- and micro topography, surface charge, porosity, wettability etc. are becoming the most important aspects for effectiveness of prosthesis biofunctionalization because of their great impact on cell adhesion, spreading, cell proliferation, differentiation and cell function. In this case choose of polymer for the scaffold becomes very important.

It is known that the set of physical, chemical and biological properties of nanofibrous scaffolds developed by electrospinning is largely determined by the fiber thickness. There are some isolated investigations aimed to find the fiber thickness for optimal physical, chemical and biological properties of PCL, PLLA, their copolymers and blends for vessel grafts [7]. Meanwhile there are no comparative studies between samples from different chemical groups that makes difficult to choose the right polymer or copolymer or blend for vessel graft development.

The aim of the study is to compare physical, topographical and biological properties of polycaprolactone, poly-L-lactic acid, polycaprolactone + poly-L-lactic acid blend, L-lactide/Caprolactone copolymer scaffolds fabricated with the same fiber thickness using electrospun technology.

2. Material and methods

Poly(ε-caprolactone) (molecular weight 70 000 g/mol) (PURASORB® PC 12, PURAC) (PCL), Poly-L-lactic acid (molecular weight 650 000 g/mol) (PURASORB® PL 38, PURAC) (PLLA) and L-lactide/Caprolactone copolymer (molecular weight 200 000 g/mol) (PURASORB® PLC 7015, PURAC) (PLC7015) were obtained from Corbion, and a Poly-L-lactic acid + Polycaprolactone blend (PCL PLLA) in a 70/30 molar ratio prepared by admixture. Solutions of the polymers in hexafluoro-2-propanol (≥99%, Sigma-Aldrich) were prepared in a hermetic glass reactor at a room temperature with a constant vortexing. Final concentrations of solutions were 7wt% for PCL, 4wt% for PLLA, 4wt% for PCL PLLA, 4wt% for PLC7015. Scaffolds were fabricated using electrospinning (NANON-1, MECC Co., Japan) at 30V potential difference using a 10x200mm (diameter x length) collector rotated at 50 rpm, 140 mm needle (22G) to target distance . Solution pump rates were optimized for each solution to achieve similar fibre thicknesses: 3 ml/hour for PCL; 5,3 ml/hour for PLLA; 5 ml/hour for PCL PLLA; 5 ml/hour for PLC7015.

Micrographs of graft morphology were obtained by means of scanning electron microscopy (JCM-6000Plus, Jeol) after gold sputtering (SmartCoater, Jeol). Resulting SEM-images were analyzed by using Image J software (National Institutes of Health, USA): fibre thickness by direct measurement, coherence using the Orientation J plugin (Biomedical Engineering group, EPFL), and pore size using a bespoke macro (Biomaterials Group, UCT) (Figure 1).

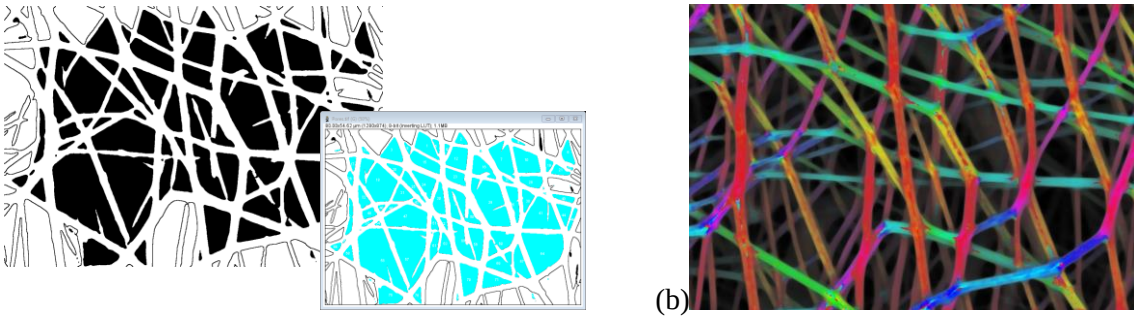

Figure 1. Example of (a) pore size quantification after elimination of edge effects and (b) false colour map assigned to fibre directions in the determination of the coherency of the scaffold fibres.

Water contact angle of the grafts surface was measured by sessile drop method (EasyDrop, Kruss). Mechanical properties of the grafts were studied under uniaxial extension using Instron 3369 installation with 50N loading cell (model 2519-102, Instron) at a loading velocity of 10 mm/min in a longitudinal direction.
For biological testing, fat-derived MMSC (multipotent mesenchyme stem cells) were collected from healthy donors and immunophenotyped with flow cytometer GuavaEasyCyte8 (Millipore, USA) using CD19, CD34, CD45, CD73, CD90 and CD105 monoclonal antibodies (BD, USA). To analyze the cell adhesion and attachment, focal adhesion vinculin was studied using fluorescence microscopy. Samples with cells after 72 hours of incubation discarded from medium, the cells washed with PBS and fixed with 4% paraformaldehyde for 20 min. Cells were permeabilised using Triton X-100 and rinsed with PBS, blocked with 10% goat serum in PBS for 30 min at room temperature, and incubated with anti-vinculin antibody (Thermo Scientific) at 1:200 dilution for 2 hours. After 3 PBS washes cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrigen) at 1:200 dilution for 1 h at room temperature in the dark. The cells were washed three times in PBS (5 min each) and stained with 4′,6 diamidino-2-phenylindole (DAPI) for nuclear visualization. Finally, the cells were washed and viewed using Carl Zeiss Axio Observer fluorescence microscope. Images were collected and processed with Zen Software. Cells were recalculated as their number per mm2.
To verify cells viability after co-cultivation with samples they were trypsinised from the material and stained with Annexin V FITC (Biolegend) and Propidium Iodide (Sigma Aldrich) for 20 minutes in the dark. Flow cytometry was performed on GuavaEasyCyte8 with detection of double positive (late apoptosis/necrosis), double negative (living) and annexin V positive (apoptosis) cells in relative count in percent. All biological tests were run at least in triplicates.
Statistical analysis was performed using Statistica 7.0 software (StatSoft, USA). Data is presented as Mean±SE (standard error of mean). The significance of difference was calculated using the Mann-Whitney U-test. Differences between groups were stated as significant in $p<0.05$.

3. Results

All developed nanofibrous scaffolds were formed by randomly entangled fibers of a cylindrical shape with a diameter of 1.2-1.3 μm and with normality in size distribution. All nanofibrous scaffolds were hydrophobic (Figure 1, Table 1) due to the low surface energy of polymers and high surface porosity [8].
The slightly lower values of contact angle for PLLA scaffolds are probably due to the greater number of functional groups on the fibers surface caused by chemical nature of the polymer material.
Scanning electron microscopy images of prepared nanofibrous scaffolds are presented in the Figure 2.

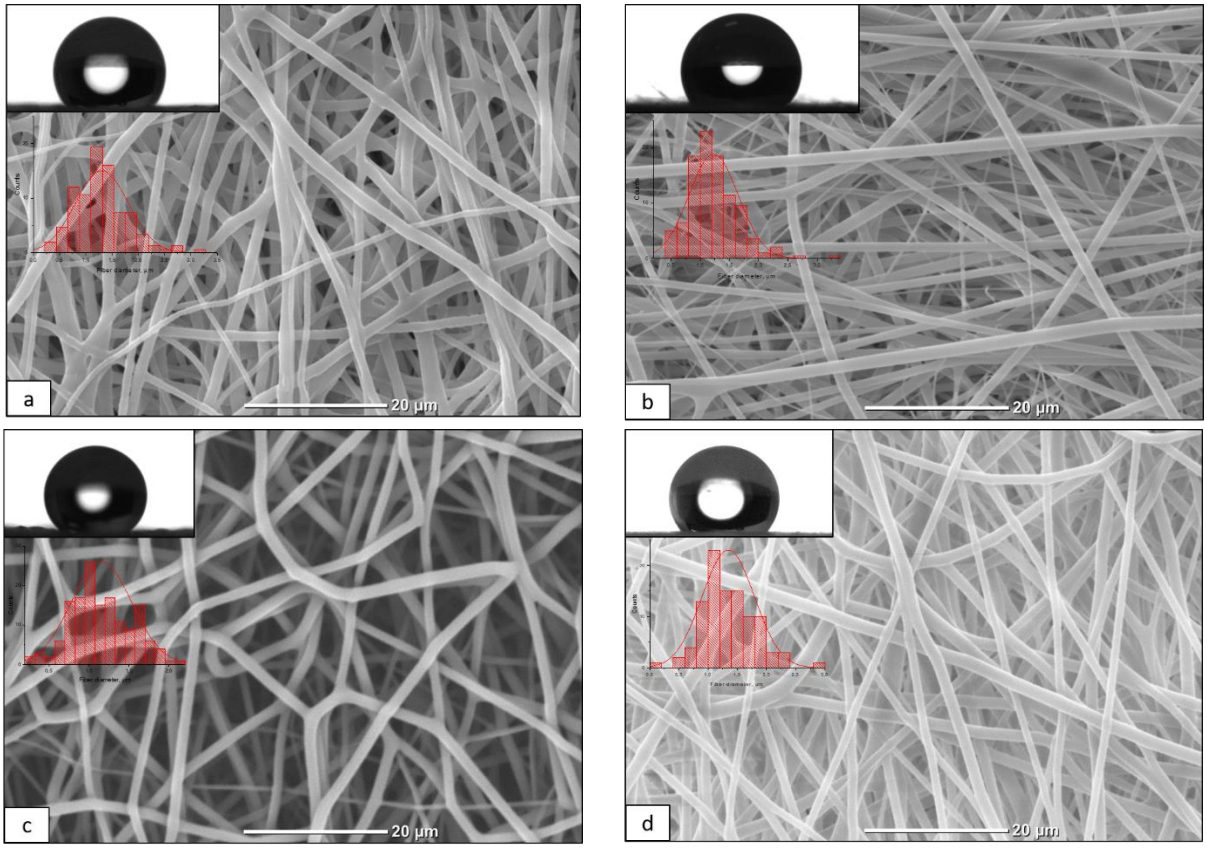

Figure 2. Scanning electron microscopy and wettability measurement of prepared nanofibrous scaffolds. A-PCL, B-PLLA, C -PCL PLLA, D-PLC7015.

The scaffolds also had similar random orientation of the fibres (average coherence = 0.13±0.06) and similar pore sizes (average 11.2±5.0 µm^2). See Table 1.

The highest strength values and the smallest value of the relative elongation at uniaxial stretching were in PLLA nanofibrous scaffolds (Table 1). It is caused by completely non-crystalline but highly oriented polymeric chains in fibers forming scaffolds [9]. PCL and PLC7015 nanofibrous scaffolds had the intermediate strength values due to the semicrystalline nature of these polymers at room temperature. Meanwhile having a lower crystallinity degree compared to PCL, PLC7015 has the higher number of passing molecules between the crystal regions, which provides the highest values of relative elongation [10][11]. The lowest strength was in PCL PLLA blend due to thermodynamic incompatibility of these polymers at the molecular level [12]. On the one hand this forms high fibers defectiveness in molecular level and on the other hand PLLA hampers the crystallization of PCL at room temperature forming a significant amount of macromolecules in the amorphous state and providing the high values of relative elongation of this type of scaffolds.

Table 1. Fiber thickness, wettability, topographical and mechanical properties of prepared nanofibrous scaffolds, Mean±SE

Material	Average fiber diameter, µm	Average pore area, µm^2	Contact wetting angle, degree	Strength, Mpa	Elastic deformation, %
PCL	1.3 ±0.5	9.5±0.6	123 ±2	8.2 ±0.7	112 ±10
PLLA	1.2 ±0.4	8.9±0.5	112 ±2[a]	13.2 ±1.0[a]	95 ±10[a]
PCL PLLA	1.2 ±0.4	18.7±1.2[a]	128 ±2	4.7±0.3[a]	265 ±44[a]
PLC7015	1.3 ±0.5	7.8±0.5[b]	123 ±2	9.1 ±1.9	560 ±157[a]

[a] p<0.01 comparing with PCL

[b] p=0.03 comparing with PCL

Materials with the same fibers thickness had different adhesion properties (Table 2, Figure 3). PLC 7015 had the lowest level of adhered MMSC and all other materials had the results as PCL. Meanwhile cell viability – parameter that basically related with chemical properties and direct toxicity – showed that PLC7015 and PCL PLLA blend had the equal percent of living cells on their surface and it was higher than those on PCL (p=0.05).

Table 2. Cell viability and cell adhesion on prepared nanofibrous scaffolds, Mean±SE.

Polymer	Cell viability, %			Cell adhesion, cells/mm^2
	Living cells	Late apoptosis/ necrosis	Apoptosis	
PCL	81.66±1.17	7.18±0.63	10.05±0.48	129±10
PLLA	77.24±0.70 [a]	11.87±1.24[a]	11.53±0.44[a]	143±10
PCL PLLA	88.36±0.32[a]	3.90±0.15[a]	6.42±0.37[a]	126±15
PLC7015	88.50±1.63[a]	4.79±0.44[a]	4.76±1.15[a]	64±3[b]

[a] p=0.05 comparing with PCL
[b] p<0.01 comparing with PCL

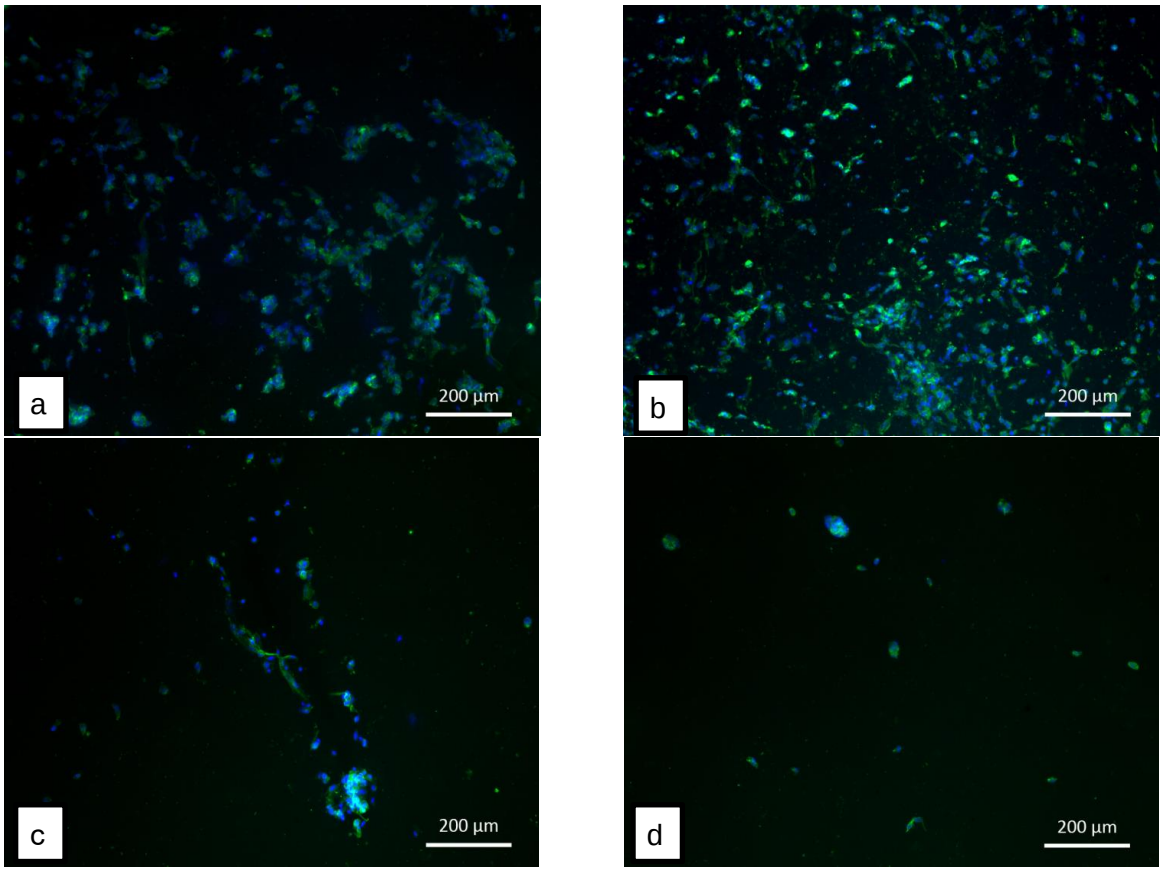

Figure 3. Adhered MMSC stained with vinculin (green) and DAPI (blue) on prepared nanofibrous scaffolds. A-PCL, B-PLLA, C -PCL PLLA, D-PLC7015.

Cells on the fibers of PCL PLLA and PLC7015 were not well spread and there were no focal adhesions (compaired to PCL and PLLA scaffolds). Taken together with adhesive cell level these data indicate that MMSC had a good first phase when the complex physicochemical interactions including hydrophobic, coulombic, and van der Waals forces between the cell membrane and the polymer surface leads to primary adhesion [3]. All interactions during this process are passive adhesion. On the next active phase arising from the integrin binding, cells start to spread and become flattened. After 72

hours of co-cultivation of MMSC with polymers surface this is observed only on the PCL and PLLA scaffolds. The third phase, when the cytoskeleton is organized to focal adhesion between cells and material was not achieved in the materials tested in this study.

4. Discussions

Different polymers, copolymers and blends could have different chemical structure which could have the impact on their properties. Besides it is proposed that nano- and micro topography, surface charge, porosity etc. are the main characteristics that directly influence on biocompatibility of the material and they are basically depended from the used manufacturing technique [3].

Synthetic biodegradable polymers such as PCL, PLLA, their blend and copolymer are the most promising materials for vascular tissue engineering and they are widely tested for different applications. Meanwhile the comparative study of electrospun materials with the close or same morphology is needed for correct chooses of the material. Besides because of high cost of copolymers comparing with blends it could be useful according to economic considerations [6].

Previously it was shown that hemolysis, coagulation pathways and thromogenicity of PCL and PLC7015 electrospun scaffolds depends on fibrous morphology and chemical composition of the scaffolds [7]. That means that adhesion properties of polyesters could be of a great addiction on material morphology in particular on fiber thickness and porosity. Also these parameters could lead to change in physical and chemical properties of the scaffolds.

Additionally, along with the material choice, it is critical to use optimal scaffold development techniques even if electrospinning is chosen as a basic technology, as the purpose is to fabricate scaffolds with polymer surfaces that mimic natural ECM (extracellular matrix) with different chemical, mechanical and topographical cues which facilitate interactions between cells and materials to control and influence cell behavior.

The impact of the chemical structure of polymers on biocompatibility is often performed using solid membranes, without taking the morphology of scaffold into account. However, by choosing the stock polymer first and comparing with others that are developed with the same ECM mimicing topography, is preferred.

Our results show that even the same topography of different polymer electrospun scaffolds could result in different biocompatibility properties. The chemical structure of the used polymers is privileged but it also means that trying to create mimic ECM one should find the balance between the polymer and optimal topography. It is also well known that surface topography influences cell adhesion, migration and proliferation. As the alignment of electrospun fibres can influence the interaction with cells, the scaffolds described in the current paper were specifically produced to have similar high randomness, as evidenced by the low coherencies of the four scaffold types. Similarly, it is evident that porosity, and specifically pore size, could influence cell adhesion and penetration, and equal care was taken to exclude this parameter as a variable.

The results show that the strength of prepared copolymer and blend scaffolds was differing than those in pure composed polymers. Copolymer PLC7015 demonstrated the same strength as PCL but the lower then PLLA. Meanwhile it has the highest level of elastic deformation. Properties of PCL PLLA blend were significantly differ then those in copolymer. The lower strength was combined with higher than in PCL scaffold elastic deformation. Actually strength of the vessel graft is a very important for long functioning in a blood flow without complications related with the hemorrhage or bleeding. Meanwhile due to elastic deformation the pulsatile wave is extinguished and it could prevent critical deformations of the graft but besides the high level of deformation could lead to aneurism of the prosthesis and to its thrombosis or disruption.

Adhesive properties and cytotoxicity of scaffolds are the main characteristics of biocompatibility for their long functioning in the blood flow. Testing these properties in vitro show the possibilities of cellularization or endothelialization of the grafts which are the first steps for biofunctionalization and future biodegradation.

Our results show that due to the used manufacturing technology and created graft topography PLC7015 copolymer have the lowest adhesive properties but adequate cytotoxicity. All other polymers and blend were rather equal in MMSC adhesion on their surfaces. We suppose such low

adhesion is due to the lower average rope area. And our preliminary results (data not shown) lead us to proposal that PLC7015 could have excellent adhesive properties which will be much more attractive then PCL or PLA itself but in the case of usage of manufacturing technique that will allow high porosity, fiber thickness, fiber heterogeneity etc.

5. Conclusion

Physical, topographical and biological properties of polycaprolactone, poly-L-lactic acid, polycaprolactone + poly-L-lactic acid blend, L-lactide/Caprolactone copolymer scaffolds fabricated with the same fiber thickness using electrospun technology could be significantly different and the chemical properties of the polymers (copolymers, blends) become the leading factor of the changed characteristics that lead to different biocompatability. Physical and topographical properties could be changed during the manufacturing process that will lead to changes in biological properties. Physical, topographical and biological properties of PCL and PLLA copolymer and blend could greatly vary from the stock polymers and these characteristics could be managed by using different electrospinning protocols.

6. References

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