J Mater Sci: Mater Med (2008) 19:1631–1636
DOI 10.1007/s10856-008-3381-x

Sterilization effects on starPEG coated polymer surfaces: characterization and cell viability

Júlia Lleixà Calvet · Dirk Grafahrend · Doris Klee · Martin Möller

Received: 17 September 2007 / Accepted: 4 January 2008 / Published online: 29 January 2008
© Springer Science+Business Media, LLC 2008

Abstract Sterilization is frequently an issue for polymeric biomaterials including hydrogels, where autoclaving needs to be discarded, and γ-irradiation and low temperature hydrogen peroxide gas plasma sterilization are already important alternatives. Coatings based on poly(ethylene glycol) are a well-known strategy to reduce unspecific protein interactions on biomaterial surfaces. Dense, ultrathin coatings of isocyanate terminated star-shaped poly(ethylene glycol) (starPEG) molecules have proven to be resistant to unspecific adsorption of proteins and enable direct biofunctionalization. The effectivity and stability of the starPEG coatings on poly(vinylidene fluoride) (PVDF) were studied after γ-irradiation (normed dosis 25 kGy) and plasma sterilization (Sterrad 100S). The selected surface properties determined were: surface composition (X-ray photoelectron spectroscopy, XPS), wettability (sessile drop contact angle) and protein adsorption by fluorescence microscopy (Avidin-TexasRed, Bovine Serum Albumin-Rhodamin). Preliminary cell experiments with the cell line L929 were performed prior and after sterilization to investigate the cell repellence of the starPEG coatings as well as cell viability and specific cell adhesion on GRGDS-modified coatings. The starPEG coating undergoes a slight oxidation due to plasma and γ-sterilization; this represents a minor variation confirmed by XPS and contact angle results. The non-sterilized starPEG and the plasma-sterilized coatings are protein repellent, however the protein adsorption on starPEG coated substrates is much stronger after γ-sterilization for both avidin and bovine serum albumin. The cell experiments indicate that the starPEG coatings are appliable homogeneously by incubation and are non-cell adherent. Moreover, after both sterilization processes the starPEG coatings remain cell repellent and the GRGDS-modified coatings presented vital cells. Thus we conclude that the plasma sterilization is more convenient for the starPEG coatings and GRGDS-modified starPEG coatings.

J. Lleixà Calvet · D. Grafahrend · D. Klee · M. Möller (✉)
DWI e. V. and Institute of Technical and Macromolecular Chemistry, RWTH Aachen Pauwelsstr. 8, 52056 Aachen, Germany
e-mail: moeller@dwi.rwth-aachen.de

1 Introduction

The unspecific adsorption of proteins onto most surfaces is being used for some applications (e.g. biocatalysts, biosensors and biochips, protein chromatography, stabilization of emulsions). However for a whole range of applications, the protein race to the surface must be prevented. Surfaces minimizing protein adsorption are necessary for enzyme linked immunosorbent assay (ELISA), sensors and contact lenses, tissue engineering, microfluidic systems, devices used for drug delivery systems for high-throughput screening using proteins or cells. The biocompatibility of biomaterials is considerably affected by the initial phenomena of protein adsorption because cells interact with surface adsorbed proteins rather than with the material surface itself [1]. Consequently, the unspecific protein adsorption leads to a variety of problems such as acute and chronic inflammation [2], fibrous encapsulation [3], occlusion of small diameter artificial blood vessels [4], complement activation [5] and biofouling of contact lenses [6].

Surface modifications preventing protein adsorption have to be hydrophilic, uncharged and flexible [7–9]. Several non-fouling polymers have achieved this

requirements: poly(hydroxyethyl methacrylate), poly(acrylamide), poly(*N,N*-dimethyl acrylamide), dextran, poly(oxazoline) and poly(ethylene glycol) (PEG). PEG is non-toxic and non-immunogenic and has been found to be the most effective of these polymers [10] and has exhibited excellent protein resistance [11]. Furthermore, starPEG systems have shown even better surface coverage if compared with linear PEG [12] and enable further functionalization of the coating (e.g. peptides, growth factors) [13].

The specific interaction of integrins with certain ECM components is a key point for biomaterial design. Therefore, the discovery that the RGD tripeptide sequence can mimic the cell attachment property of the whole protein fibronectin [14] is an important advance.

Biomedical devices and surgical instruments for tissue-engineering or clinical applications must be either manufactured aseptically or sterilized before use. Plasma-based methods, ionizing radiation, and other advanced technologies have recently been applied to low-temperature sterilization and decontamination. For example, the low-pressure glow discharge plasma is employed in combination with a gas such as hydrogen peroxide, which is effective against a broad range of bacteria and bacterial spores. The microorganisms are killed by the generated oxygen, hydroxyl free radicals and other active species, although these mechanisms are still being investigated [15, 16].

Plasma sterilization is a non-toxic, fast procedure without the severe disadvantages of other methods when dealing with thermolabile materials. Plasma sterilization has been proven to be a microbiologically safe procedure with legally adequate reduction factors of >6 log [17]. Compared to other sterilization methods such as ethylene oxide (ETO), plasma sterilization not only kills the bacteria and viruses, but also removes the dead pyrogens and their debris from the surface, because the sterilization process is similar to plasma etching [16]. Among new plasma low-temperature sterilization methods, the Sterrad gas-plasma technique employing hydrogen peroxide has similar sterilization efficiency to that of ETO and γ-irradiation [18, 19].

2 Materials and methods

PVDF foils were purchased from Good Fellow (England). All solvents and buffers are from Sigma–Aldrich (Germany). The dye fluorescein isothiocyanate (FITC) was from Fluka (Germany). Fluorescence labeled proteins (Avidin-TexasRed and Bovine Serum Albumin-Rhodamin) and GRGDS were purchased from Bachem (Switzerland) and live/dead viability/cytotoxicity kit from Invitrogen (Germany). Cell line L929 was kindly donated by Uniklinikum Aachen. Plasma sterilization was performed in a 100S model (Sterrad), γ-sterilization was carried out at normed dosis 25 kGy according to EN 552.

In order to generate amino groups for the hydrogel immobilization the foils were treated with NH_3 plasma. This surface activation was accomplished in a low-pressure low temperature microwave plasma machine from Roth and Rau (Germany). Contact angle and XPS measurements were made to quantify the effect of the plasma activation. Fluorescence microscopy with FITC as dye was used to estimate qualitatively the homogeneous generation of amino-groups on the sample surface. Sessile drop contact angle measurements were made with goniometer G402 from Krüss (Germany). Average contact angles of 10 independent measurements are presented. XPS spectra were carried out in a Ultra Axis^{TM}-Spektrometer from Kratos Analytical (UK). The at% presented is an average of three measurements. The samples were incubated for 1 h in 1 ml of bidistilled water with 50 µl of a 1 mg FITC/ml abs. DMSO solution and were therein after washed five times in 1 ml bidistilled water and dried in a N_2-flow. Afterwards they were examined using an Axioplan 2 imaging microscope (Zeiss).

To prepare the hydrogel coating, 10 mg/ml starPEG (M_n = 12 kDa) were solved in a water 9:1 tetrahydrofuran (v/v) mixture. Sessile drop contact angle (n = 10), XPS (n = 3) and fluorescence microscopy were performed with the equipment described previously. To investigate the protein adsorption, the samples were immersed for 20 min in 1 ml of a solution containing 5 µg/ml in PBS-buffer (pH 7.4) of the corresponding marked protein. Subsequently the samples were washed twice gently for 20 min in bidistilled water and were finally dried in a N_2-flow.

GRGDS was coupled by 12 h incubation consecutively after starPEG coating (concentration 50 µg/ml) in sodium hydrogen carbonate buffer (0.1 M, pH 8.4). Cell suspension had 60,000 cells/ml (RPMI cell media, PAA). All samples were pre-seeded under the clean bench for 1 h and were incubated for 24 h after adding cell media. The live/dead viability/cytotoxicity kit was applied as defined in the product instructions. The samples were observed under the fluorescence microscope mentioned previously. For cell experiments, three replicates per sample type were produced. The statistics are the cell density counted on five squares of 1 mm^2 taken randomly per each sample.

3 Results and discussion

Enhanced biocompatibility and performance of PVDF foils can be achieved by modifying the surface with a starPEG coating showing non-fouling properties and peptide

sequences targeting the integrin receptors present on cells. A process was developed to apply these coatings to PVDF foils containing several steps. The corresponding steps were monitored by XPS measurements, contact angle measurements, protein adsorption and cell culture experiments. Two fluorescence labeled model proteins, bovine serum albumin and avidin, were chosen to investigate the adsorption behavior on the samples. The cell line L929 are standard mouse fibroblast commonly used for biocompatibility studies.

In order to coat the PVDF foils with starPEG layers, amine groups have to be generated on the surface, which later are used for the covalent immobilization of the ultrathin starPEG coating. FITC is a fluorescence dye used for the qualitative visualization of the generated amine groups because it typically couples to primary amines. Although the PVDF foils showed autofluorescence, the successful introduction of amino groups on the surface of these foils is demonstrated by the qualitative increase in fluorescence intensity seen in Fig. 1. XPS measurements confirm the generated amine groups with a nitrogen content of 3.3 at%. The induced amine groups result in a slight hydrophylization of the surface that is detected by the contact angle data.

After surface activation, the starPEG coating was applied to the PVDF foils by incubation. The successful immobilization of the coating was observed by the change in surface composition determined by XPS, which is close to the values for ultrathin starPEG coatings [20] (see Table 1). The protein repellent properties of the starPEG coating are shown in Fig. 2. Independently of the used model protein, suppressed unspecific adsorption was observed on the starPEG modified surfaces.

Prior to implantation, biomaterials need to be sterilized. Sterilization remains a critical step for surface modifications such as hydrogel coatings and immobilized biological active molecules, and the effects of different methods on the integrity of the surface modification need to be investigated. The non-fouling properties of the starPEG layer need to be conserved, while the immobilized GRGDS must

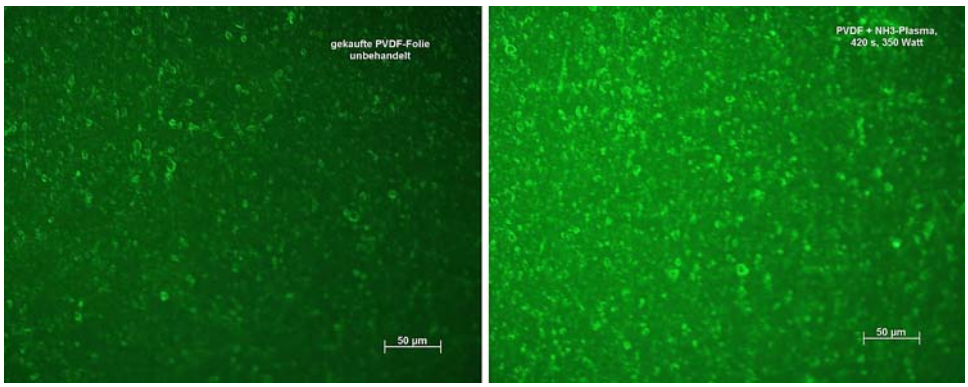

Fig. 1 Amine group detection on PVDF substrates using FITC as dye before and after plasma activation with NH_3 gas by low temperature low pressure plasma treatment (10×)

Table 1 XPS and contact angle data for untreated, NH_3-plasma activated and starPEG coated PVDF foils prior and after plasma and γ-sterilization.

	XPS (at%)								Contact angle (°)
	C1s					O1s	N1s	F1s	
	Σ	285.0 eV C–H, C–C	286.5 eV C–O, C–N, C–F	287.7 eV R–C=O, O–C=O	290.5 eV R–COOH, CH_2-CF_2	Σ	Σ	Σ	
Untreated	52.4 (±2.1)	7.5 (±2.1)	23.8 (±0.5)	10.1 (±10.9)	10.9 (±9.4)	4.4 (±1.1)	–	41.7 (±1.8)	77.3 (±0.9)
NH_3-plasma activated	78.8 (±1.4)	54.4 (±2.2)	12.2 (±0.9)	4.7 (±2.3)	7.6 (±4.6)	4.3 (±1.0)	3.3 (±0.3)	13.1 (±1.6)	66.0 (±1.9)
StarPEG unsterile	72.7 (±1.1)	23.6 (±2.4)	36.0 (±1.2)	5.1 (±6.0)	8.0 (±6.9)	11.6 (±1.5)	2.7 (±0.1)	12.4 (±0.8)	70.7 (±1.4)
StarPEG plasma sterilized	71.5 (±0.5)	20.1 (±3.2)	34.9 (±1.5)	2.9 (±0.8)	14.2 (±1.5)	7.8 (±0.6)	2.4 (±0.6)	18.2 (±0.8)	58.7 (±1.8)
StarPEG γ-sterilized	69.0 (±2.2)	19.8 (±1.0)	30.0 (±0.5)	2.2 (±0.9)	16.9 (±0.4)	8.2 (±0.6)	2.3 (±0.4)	18.2 (±2.9)	65.2 (±2.0)

Standard deviations are included in brackets

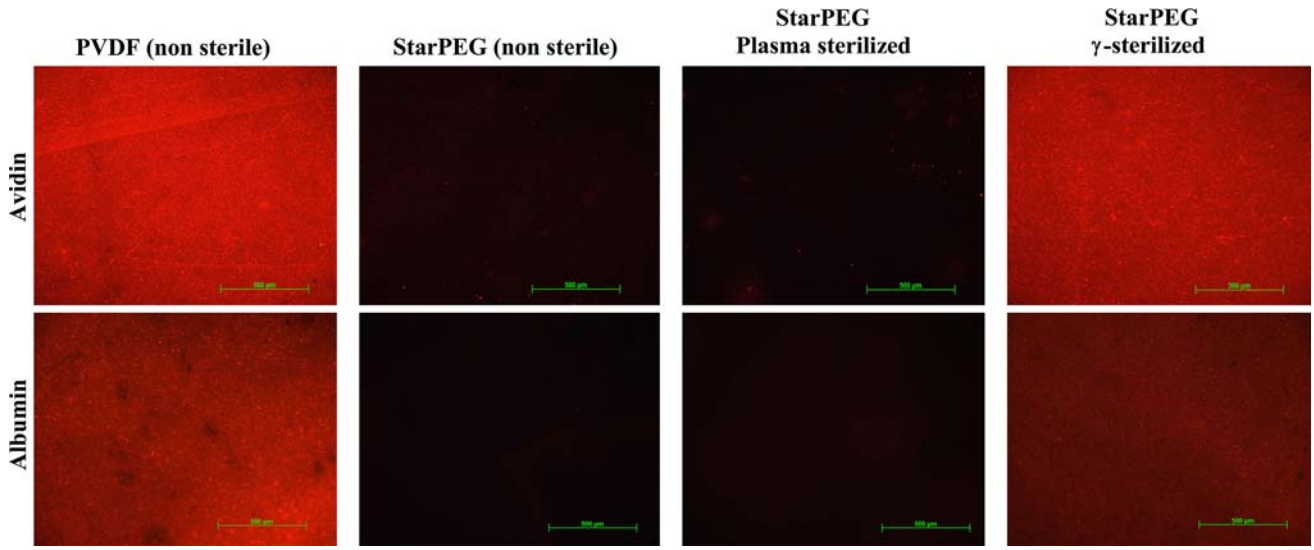

Fig. 2 Protein adsorption of Avidin-TexasRed and Bovine Serum Albumin–Rhodamin on starPEG coated PVDF foils (left column: reference—non-coated PVDF). Exposure time 15 s (5×)

remain active. Two sterilization methods, plasma sterilization and γ-sterilization, were used and the effects of both methods studied.

Both sterilization methods altered the surface properties of the starPEG coated samples in terms of surface composition and wettability. After either sterilization methods, it is possible to identify a small reduction in total carbon and nitrogen content as well as a significant decrease in total oxygen content and an enhanced rise in fluor content (from 12.4 to 18.2 at% after both sterilization methods). The plasma sterilization results in a hydrophylization from 70.7° to 58.7° of the starPEG coated surface, whereas only minor changes were observed in the case of γ-sterilization.

The sterilization methods proved to have impact on the integrity of the protein repellent starPEG layer to different extents (see Fig. 2). While plasma sterilized starPEG coated PVDF foils remained protein repellent, γ-sterilized samples showed adsorption of both model proteins. A homogeneous red fluorescence indicates that the γ-sterilization has affected the starPEG coating overall.

Although the effects of both sterilization methods on the protein adsorption are visible, it was still to be evaluated if this influences would be correlated to the in vitro cell response. Figures 3 and 4 present the cell surface density and cell viability respectively on the different PVDF foils before and after sterilization. Ethanol 70% (v/v) disinfection was chosen as a reference disinfection method known for being non-invasive for the starPEG coatings. Green fluorescence indicates the viable cells while red fluorescence is a sign for cell apoptosis.

Non-modified PVDF foils provide good substrates for the L929 mouse fibroblasts, indicated by the high viable

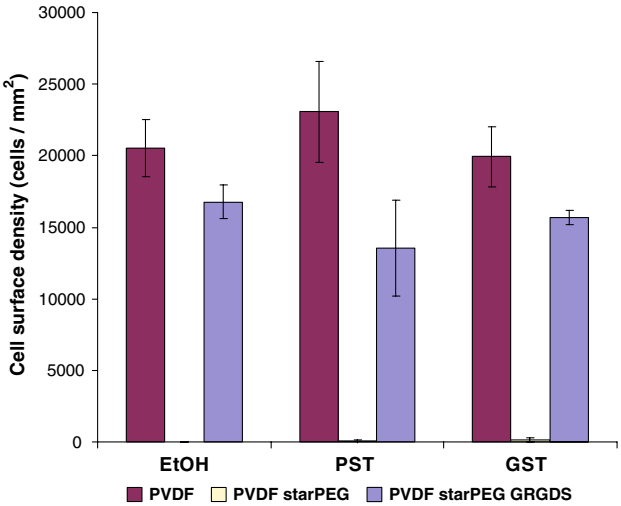

Fig. 3 L929 cell surface density [cells/mm^2] on PVDF, starPEG coated and GRGDS modified starPEG coated PVDF foils after ethanol 70% disinfection (EtOH), plasma sterilization (PST), and γ-sterilization (GST). Error bars are standard deviations

cell density (see Fig. 3). In contrast to this, all starPEG coated PVDF foils show almost no cells showing that cell repellence is conserved independently of the disinfection or sterilization method. Even if differences in the protein repellence properties of these samples depending on the sterilization method were observed, these tendencies were not confirmed by the in vitro tests. Cells could not attach to the starPEG coated surfaces after plasma-sterilization and γ-sterilization. Immobilized GRGDS on the starPEG coated PVDF foils conserved its activity and led to high viable cell densities. Generally, the highest cell densities

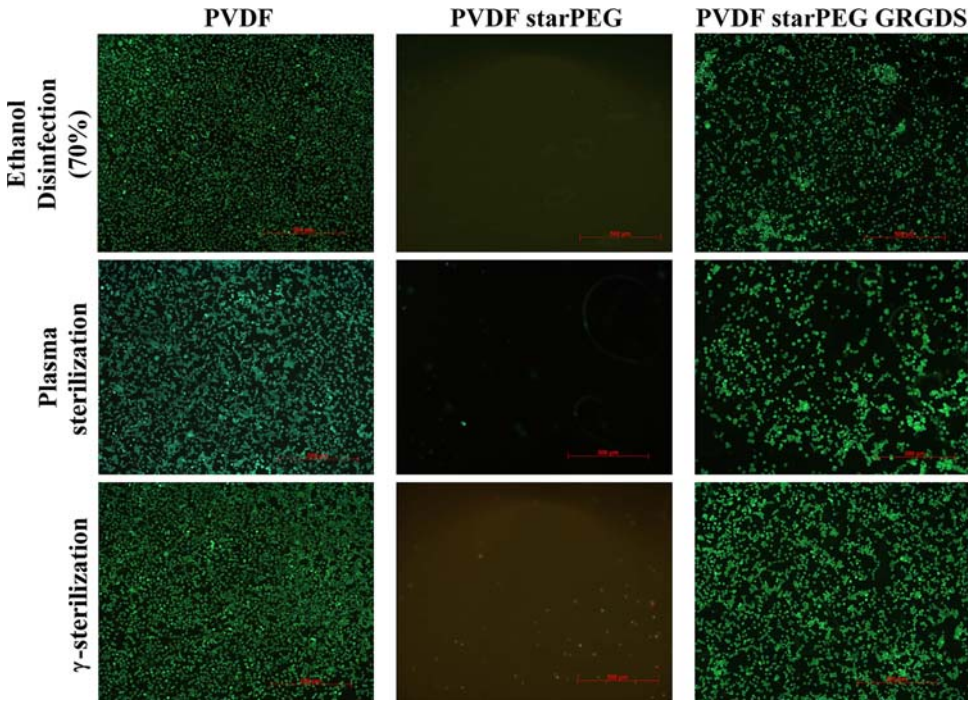

Fig. 4 Cell line L929 on PVDF (*left*), PVDF with starPEG (*middle*) and PVDF with starPEG and GRGDS (*right*)—samples were disinfected with ethanol 70% (*upper*) as reference and plasma (*middle*) and γ-sterilized (*down*) (5×)

were observed on non-modified PVDF foils. While in this case unspecific cell attachment is observed, cell attachment on the GRGDS immobilized starPEG coated PVDF foils is actively induced by the peptide sequence. As expected, among the peptide immobilized starPEG coated PVDF foils, the samples which were disinfected with ethanol showed the highest cell densities. Cell densities on γ-sterilized substrates were comparable to this reference, while plasma sterilization resulted in the lowest cell densities.

4 Summary

The starPEG coating undergoes a slight oxidation due to plasma and γ-sterilization; this represents a reduction in total oxygen content but a notable increase of carboxylic groups from the carbon. This is confirmed by the minor hydrophilization after sterilization, with respect to the non-sterilized starPEG coated PVDF.

The protein adsorption on starPEG coated substrates is much stronger after γ-sterilization for both avidin and bovine serum albumin. The non-sterilized starPEG and the plasma-sterilized coatings are protein repellent.

The protein adsorption and cell experiments demonstrate that the starPEG coatings are appliable homogeneously by incubation and are non-cell adherent. Moreover, after both sterilization processes the starPEG coatings remain cell repellent and the GRGDS-modified coatings present vital cells. The GRGDS-modified starPEG coatings still promote cell adhesion after both sterilization processes.

Resumedly, γ-sterilization results in unspecific protein adsorption, which leads us to resolve that plasma sterilization has a minor effect on the starPEG layers. We suggest that the plasma sterilization may etch the starPEG coating whereas the γ-sterilization could cleave partially the starPEG network in the hydrogel layer. Altogether we conclude that the plasma sterilization should be favored in opposition to γ-sterilization for starPEG coatings.

Acknowledgments This work was supported by BMBF project Nr. 0330469. Júlia Lleixà Calvet would like to thank Alexandra Goldyn for her instruction in cell experiments. SusTech Darmstadt is acknowledged for support with the StarPEG system.

References

1. P. Cacciafesta et al., Langmuir **16**, 8167 (2000)
2. L. Tang et al., PNAS **95**, 8841 (1998)
3. R. Johnson et al., Biomed. Sci. Instrum. **34**, 47 (1997)
4. G.W. Bos et al., J Biomed. Mater. Res. **44**, 330 (1999)
5. A. Kidane et al., J. Biomed. Mater. Res. **44**, 640 (1999)
6. P. Kingshott et al., Anal. Biochem. **273**, 156 (1999)
7. W. Norde, Macromol. Symp. **103**, 5 (1996)
8. J. Buijs, V. Hlady, J. Colloid. Interface Sci. **190**, 171 (1997)
9. J.N. Turner, W. Shain, Exp. Neurol. **156**, 33 (1999)
10. J.M. Harris, (ed.), *Poly (ethylene glycol) Chemistry: Biotechnical and Biomedical Applications* (Plenum Press, New York, 1992)
11. S.I. Jeon et al., J. Colloid. Interface Sci. **142**, 149 (1991)
12. J. Groll et al., Biomacromolecules. **6**, 956 (2005)
13. J. Groll et al., J. Biomed. Mat. Res. Part A **74A**(6), 607 (2005)

14. M.D. Pierschbacher, Ruoslahti E., Nature **309**, 30 (1984)
15. M.D. Pierschbacher, Ruoslahti E., Nature **309**, 30 (1984)
16. T.C. The et al., Biomaterials **17**(13), 1273 (1996)
17. A.A. Bialasiewicz et al., Ophtal. Res. **27**(1), 124 (1995)
18. K. Kelly-Wintenberg et al., J. Vac. Sci. Technol. A **17**(4), 1539 (1999)
19. C.E. Holy et al., Biomaterials **22**(1), 25 (2001)
20. E.A. Amir et al., J. Chem. Phys. Chem. **5**, 552 (2004)