Alexander Rudt*, Tonya D. Andreeva, Stefka G. Taneva, Rumen Krastev

Composite polyelectrolyte multilayers for biofunctionalization of medical devices

Abstract: Polyelectrolyte multilayer coatings (PEM) are prepared by alternative layer-by-layer deposition of cationic and anionic polyelectrolyte monolayers on charged surfaces. The thickness of the coatings ranges from nm to few µm. Their properties such as roughness, stiffness, surface charge and surface energy can be precisely tuned to fulfill different technical or biological requirements. The coating process is based on self-assembly of polyelectrolytes. Advantages of these coatings are their easy handling, no harsh chemistry and the possibility for coatings on complex geometries. The PEM coatings can be prepared from a variety of suitable polyelectrolytes. Their stability varies from very durable PEM coatings that are only soluble in strong solvents to quickly degradable, which may be applied as drug release system. One example of such a degradable PEM system is the one based on the polyelectrolyte pair Hyaluronan (HA) and Chitosan (CHI). These biopolymers originate from natural sources and show low toxicity towards human cells. However, HA/CHI multilayers shows only weak adhesiveness for human umbilical vein endothelial cells (HUVEC). In this article, we summarize our approaches to enhance the HA/CHI multilayer by incorporation of a non-polymer substance –graphene oxide– to improve the cell adhesion and keep such properties as low cytotoxicity and biodegradability. Different approaches for incorporation of graphene oxide were performed and the cellular adhesion was tested by metabolic assay.

Keywords: polyelectrolyte multilayers, biocompatibility, cell adhesion, composite films, graphene oxide

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***Corresponding author: Alexander Rudt:** Reutlingen University, Reutlingen, Germany, E-Mail: alexander.rudt@reutlingen-university.de
Tonya D. Andreeva, Rumen Krastev: Reutlingen University, Reutlingen, Germany and NMI Natural and Medical Sciences Institute at the University of Tübingen, Reutlingen, Germany
Stefka G. Taneva: Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, Sofia, Bulgaria

1 Introduction

The demand for development of new materials that are in contact with biological tissues and fluids is steadily rising. In recent years, the construction of polyelectrolyte multilayers (PEM) has evolved as a universal strategy to engineer surface coatings for biofunctionalization and drug delivery between non-viable biomaterials and vital biological tissues. One of the advantages of these multilayers is the possibility to control their structure and properties with high precision to meet the requirements of biological tissues. Although a large number of polymers have already been utilized as suitable materials for implants and their coatings, many induce a strong inflammatory and thrombogenic response. There is a demand for optimization of PEM coatings as well as for better understanding of the interactions between materials and biological systems.

The objective of this paper is the elaboration of composite PEM films from Hyaluronan (HA) and Chitosan (CHI) with incorporated graphene oxide (GO). These composite coatings possess well-defined and controllable physicochemical surface properties and tunable interactions with biological cells. The polyelectrolyte pair hyaluronan/chitosan (HA/CHI) is selected as one of the most promising for the functionalization of implants, as these biopolymers are biodegradable and non-toxic. However, these coatings are not suitable to promote cellular adhesion [1].

There are various approaches to modify the surface properties of PEMs and hence their biological response. One strategy is the addition of non-polymeric components on top or in-between PE-layers. Herein we present/report the effect of incorporation of graphene oxide in various localizations and proportions into the HA/CHI films, on their properties and on the adhesion of Human umbilical vein endothelial cells (HUVECs). Graphene oxide, an ultra-thin two-dimensional nanomaterial rich in oxygen-containing functionalities, has already been applied for biomedical application, particularly for drug-delivery systems. The application of graphene oxide is promising, as it demonstrates low toxicity and e.g. anticancerous activity when combined with silver [2].

2 Experiment

2.1 Preparation and characterization of PEM films

All PEM films were prepared on glass slides by applying the Layer-by-Layer (LbL) technique. The polyelectrolytes were dissolved in 250 mM NaCl solution with pH 5.5. GO was applied as a water dispersion with concentration of 0.5 mg/ml. The substrates were sequentially dipped for 10 min in solutions of polycation (CHI), polyanion (HA) and graphene oxide (GO). Each deposition step was followed by rinsing in fresh solution of 250 mM NaCl (three times for 2 min). After the last deposition step, the samples were washed in ultra-pure water and dried in nitrogen.

The following composition of PEM films with graphene oxide were studied:

bPEM: PEI/$(HA/CHI)_{10}$
basic control film without GO;

hPEM1: PEI/$(HA/CHI)_9$/GO
hybrid film with one surface exposed GO-layer;

hPEM2: PEI/$(HA/CHI)_9$/$(GO/CHI)_1$
hybrid film with one embedded GO-layer;

hPEM3: PEI/$(HA/CHI)_2$/$(GO/CHI)_7$/$(HA/CHI)_1$
hybrid film with seven embedded GO-layers.

The PEM coatings were characterized by ellipsometry (Sentech, Germany) and static contact angle measurements (Krüss, Germany) applying Young-Laplace fitting procedure. Atomic force microscopy (JPK, Germany) was utilized for monitoring the surface topography and for calculating the surface roughness. Peak-to-valley roughness (Rz), which is the arithmetic mean of the five highest peaks and the five deepest valleys over the evaluated area, was used to characterize the surface roughness.

2.2 Cell culture and cell vitality assay

Human umbilical vein endothelial cells (HUVECs) are human primary cells that are known for their clinical significance and high sensitivity to the surrounding. The HUVECs were seeded and cultured for 48 h on PEM coated substrates and non-coated substrates used as controls. The positive control was a commercial tissue culture treated polystyrene well plate (Corning, USA). The negative control was an uncoated polystyrene well plate (Greiner Bio-One, Germany). The amount of vital adhered cells can be made visible with resazurin, which is reduced by Cytochrome and e.g. NADPH/NADP, FADH/FAD resulting in colorimetric change that correlates with the quantity of cells in question.

After cultivation of HUVECs for 48 h, the culture medium was replaced with medium containing resazurin. A defined incubation time allowed the viable cells to reduce resazurin to the fluorescent resorufin, which was quantitatively measured by fluorescence spectroscopy.

3 Results and conclusion

The thickness, contact angle and roughness of the PEM coatings without and with incorporated GO were measured and the results are summarized in Table 1. All tested coatings have similar thickness of around 90 nm. The substitution of polymer layers with GO-layers shows no effect on thickness. The roughness Rz is comparably low (~15 nm) with the exception of the hybrid PEM with seven incorporated GO layer (**hPEM3**) , which is around 100 nm.

Table 1: Thickness, static contact angle and roughness Rz of the studied PEMs with incorporated GO.

PEM	Thickness, nm	Contact angle, degrees	Roughness Rz, nm
bPEM	94.0 ± 2.3	57.9 ± 1.9	13.7 ± 4.0
hPEM1	84.4 ± 3.0	40.7 ± 2.1	17.7 ± 1.4
hPEM2	88.4 ± 3.0	49.6 ± 2.9	15.5 ± 0.9
hPEM3	90.0 ± 2.5	37.2 ± 2.3	100.2 ± 15.2

The surface wettability is considered a fundamental property of each coating, because it is responsible for the adsorption of proteins and other contacting molecules, and for adhesion of cells and microorganisms [3]. The basic control PEM film is hydrophilic, with a contact angle of ~58 °. All hybrid films are more hydrophilic. Since GO is hydrophilic due to the presence of oxygen functionalities, the contact angles of hybrid PEMs correlate to the ones reported for GO in the literature (46.5º according to [4], 36.4º according to [5]).

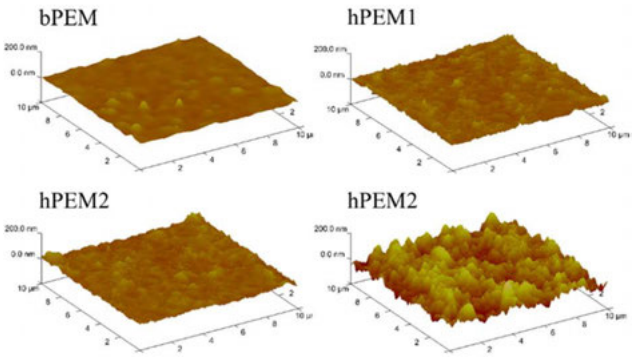

Figure 1: Topography of PEMs without and with incorporated GO studied by atomic force microscopy

The incorporation of GO-layer(s) in the PEM reshapes its surface morphology and hence the surface roughness. When GO is deposited as a top layer, the surface roughness of the hybrid **hPEM1** film is similar to that of the control film without GO (**bPEM**). This fact supports the assumption that GO-sheets follow the morphology of the underlying layer, based on AFM-imaging (Figure 1). The incorporation of only one GO-layer into the polymer matrix does not affect the surface roughness. However, the topography changes and Rz increases with the number of incorporated GO-layers, and for **hPEM3** it is 11-fold higher than that of the basic control PEM film (**bPEM**), revealing the presence of high peaks and deep valleys.

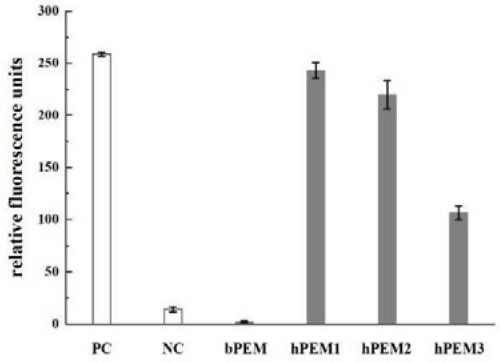

Figure 2: Biological characterization of PEMs without and with incorporated GO and positive and negative controls by resazurin assay

Figure 2 demonstrates that the adhesion of HUVECs on the control film without GO (**bPEM**) is very low, similar to the negative control (untreated polystyrene). It has already been demonstrated that biofunctionalization of NiTi surface with HA/CHI films reduces the platelets adhesion by 38 % compared to the bare NiTi surface [6]. Most polysaccharide multilayer films containing more than six bilayers exhibit poor or even anti-adhesive properties, presumably due to their high hydration and low elastic modulus.

On the contrary, on the **hPEM1** film with surface exposed GO-layer the adhesion of HUVECs is higher and comparable to that on the positive control. Deposition of just one additional CHI-layer on GO-layer (**hPEM2**) reduces the number of adhered cells by about 10 %. The film **hPEM3** with seven incorporated GO-layers and with one HA/CHI bilayer on top reduces HUVEC adhesion by 56 %.

In conclusion, the hPEM1 and hPEM2 coatings are certainly improving the cytophilicity of the antiadhesive uncoated polystyrene. The basic control coating without GO (**bPEM**) shows very poor cellular adhesion, which is comparable to the negative control.

It is known that cell adhesion is complex phenomena depending on numerous factors such as surface charge, stiffness, hydrophilicity and roughness [7]. Our results demonstrate that HUVEC adhesion does not depend on the thickness and hydrophilicity of the PEM films, because all films are hydrophilic and their thickness is similar. Although the roughness of **hPEM3** is much higher than that of **bPEM** and **hPEM1** films which have similar roughness. The three hybrid films show very different cell adhesion.

It appeared that the HUVECs adhesion on the hybrid HA/CHI/GO films originates from the concurrent impact of two factors – the number of GO-layers and their location into the polymer matrix. Incorporation of GO-layer closer to the surface of the film increases its ability to adhere cells. This is the factor behind the arrangement of the pro-adhesive properties of the hybrid films in the order bPEM □ negative control < hPEM3 < hPEM2 < hPEM1 □ positive control. It is known that GO-layers exhibit very high stiffness (Young's modulus ~200 GPa [8]), while the strongly hydrated HA/CHI multilayers are quite soft (Young's modulus ~150 kPa [9]), hence the stiffness of the hybrid HA/CHI/GO films should increase both with the number of embedded GO-layers and with their incorporation them closer to the surface, similar to what was reported for PSS/PAH/GO films [10].

Therefore, the difference in the HUVECs adhesion originates from the changes in film rigidity, triggered by the introduction of GO-layers, being most favorable for the **hPEM1** multilayer with surface exposed GO-layer. Further studies need to be performed to clarify the exact reason behind adhesion-promoting property of GO incorporated in antiadhesive PEM systems, such as screening of stiffness over depth.

4 Summary

Polyelectrolyte multilayers formed from Hyaluronan (HA) and Chitosan (CHI) are biocompatible, non-cytotoxic and biodegradable, hence are promising for application as coatings for medical products with optional drug release. However, adhesion of human cells such as primary endothelial cells on HA/CHI coatings is poor, which would hinder healthy ingrowth of implants and complicate the application of these coatings on implants.

In this work we show that incorporation of graphene oxide in different locations and proportions into the HA/CHI PEM coatings enables controllable adhesion of primary human endothelial cells (HUVECs) on these surfaces. The

incorporation of GO into HA/CHI multilayers does not influence the thickness and hydrophobicity of the coatings, but has an impact on surface stiffness and roughness. The improved cellular adhesion on the hybrid PEM coatings is most probably caused by the altered surface stiffness. Further studies need to be carried out for in depth analysis of the composite film properties on the cellular behavior.

Author Statement

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References

[1] Richert L, Lavalle P, Payan E, Shu XZ, Prestwich GD, Stoltz JF, Schaaf P, Voegel JC, Picart C. Layer by layer buildup of polysaccharide films: physical chemistry and cellular adhesion aspects. Langmuir 2004 Jan 20;20(2):448–58.

[2] Kavinkumar T, Varunkumar K, Ravikumar V, Manivannan S. Anticancer activity of graphene oxide-reduced graphene oxide-silver nanoparticle composites. J Colloid Interface Sci 2017 Nov 1;505:1125–1133.

[3] Choee JH, Lee SJ, Lee YM, Rhee JM, Lee HB, Khang G. Proliferation rate of fibroblast cells on polyethylene surfaces with wettability gradient. J Appl Polym Sci 2004;92(1):599–606.

[4] Panda D, Nandi A, Datta SK, Saha H, Majumdar S. Selective detection of carbon monoxide (CO) gas by reduced graphene oxide (rGO) at room temperature. RSC Adv 2016;6:47337–47348.

[5] Lee J, Atmeh M, Berman D. Effect of trapped water on the frictional behavior of graphene oxide layers sliding in water environment. Carbon 2017;120:11–16.

[6] Thierry B, Winnik FM, Merhi Y, Silver J, Tabrizian M. Bioactive coatings of endovascular stents based on polyelectrolyte multilayers. Biomacromolecules. 2003 Nov–Dec;4(6):1564–71.

[7] Gribova V, Auzely-Velty R, Picart C. Polyelectrolyte multilayer assemblies on materials surfaces: from cell adhesion to tissue engineering. Chem Mater. 2012 Mar 13;24(5):854-869.

[8] Suk JW, Piner RD, An J, Ruoff RS. Mechanical properties of monolayer graphene oxide. ACS Nano 2010, 4, 11, 6557–6564.

[9] Andreeva TD, Hartmann H, Taneva SG, Krastev R. Regulation of the growth, morphology, mechanical properties and biocompatibility of natural polysaccharide-based multilayers by Hofmeister anions. J. Mater. Chem. B, 2016, 4, 7092–7100.

[10] Qi W, Xue Z, Yuan W, Wang H. Layer-by-layer assembled graphene oxide composite films for enhanced mechanical properties and fibroblast cell affinity. J. Mater. Chem. B, 2014, 2, 325–331.