Chemical Design of Non-Ionic Polymer Brushes as Biointerfaces: Poly(2-oxazine)s Outperform Both Poly(2-oxazoline)s and PEG

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Abstract: The era of poly(ethylene glycol) (PEG) brushes as a universal panacea for preventing non-specific protein adsorption and providing lubrication to surfaces is coming to an end. In the functionalization of medical devices and implants, in addition to preventing non-specific protein adsorption and cell adhesion, polymer-brush formulations are often required to generate highly lubricious films. Poly(2alkyl-2-oxazoline) (PAOXA) brushes meet these requirements, and depending on their side-group composition, they can form films that match, and in some cases surpass, the bioinert and lubricious properties of PEG analogues. Poly(2-methyl-2oxazine) (PMOZI) provides an additional enhancement of brush hydration and main-chain flexibility, leading to complete bioinertness and a further reduction in friction. These data redefine the combination of structural parameters necessary to design polymer-brush-based biointerfaces, identifying a novel, superior polymer formulation.

The biopassivity and lubrication of molecularly tailored surfaces have recently been gaining particular industrial interest as both of these characteristics are fundamental to the design of many biomedical and biomechanical devices,

such as articular prostheses, catheters, intraocular lenses, and biosensors.

As many inorganic and organic materials used for these applications present a negatively charged oxide interface at physiological pH values, comb-like or graft copolymers featuring a polycationic backbone and bioinert side chains represent a highly versatile, robust, and broadly applicable solution to the fabrication of brush assemblies through simple dip-and-rinse processes, simultaneously preventing protein contamination and reducing friction.[1–6]

In particular, poly(l-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) has been applied to form lubricious and antifouling PEG brush coatings on metal oxide surfaces with low isoelectric points,[7–10] as well as on polymeric supports,[11–13] while analogous graft copolymers featuring PEG bioconjugates have been successfully employed to support cell adhesion and proliferation on a variety of

surfaces.[11,14,15]

Despite the broad applicability of PLL-g-PEG films, several drawbacks have been associated with the application of PEG derivatives, including their tendency to undergo oxidative degradation to yield toxic compounds,[16,17] and the expression of antibodies specific for PEGs in vivo.[18–20] Appropriate alternatives that display improved chemical stability would be highly desirable, while polymers presenting more easily tailorable chemistries would give access to a larger variety of functional surfaces.

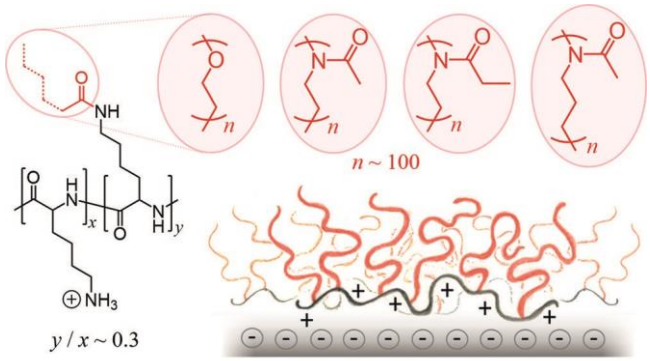
Poly(2-alkyl-2-oxazoline)s (PAOXAs), in particular the hydrophilic variants poly(2-methyl-2-oxazoline) (PMOXA) and poly(2-ethyl-2-oxazoline) (PEOXA), have been emerging as very promising alternatives to PEGs in a variety of biotechnological applications, showing comparable

physicochemical properties, biocompatibility, and stealth properties.[21–27] Moreover, PAOXA films on organic and inorganic supports showed similar biopassivity to their PEG counterparts,[28–31] and a significantly higher resistance towards
oxidation.[32]

Following these pioneering reports, the need for a general, comparative study of the properties of different PAOXA brushes and their PEG analogues has emerged, stimulating the present work. In addition to investigating the properties of
PMOXA and PEOXA brushes, we expanded our study to include poly(2-methyl-2-ozazine) (PMOZI) grafts, which are isomeric to PEOXA, but present a methyl group as a side chain (as in PMOXA) and contain one additional methylene function along the repeating unit.

As with PAOXAs, poly(2-alkyl-2-oxazine)s (PAOZIs) are synthesized by cationic ring-opening polymerization (CROP), starting from five-membered or six-membered cyclic imino ether monomers, respectively,[33–35] enabling a controlled polymerization process, and the facile preparation of multifunctional polymers. As PMOZI features very similar chemical traits to PMOXA and PEOXA, but has a different structural arrangement within the monomer unit, it is a particularly interesting polymer for evaluating the effects of the macromolecular architecture on the physicochemical properties of the subsequently generated brushes.

In this study, we systematically compare the interfacial physicochemical properties of brushes generated from PLLg-PMOXA, PLL-g-PEOXA, and PLL-g-PMOZI on SiO_2 surfaces, and correlate them to those displayed by the "gold standard" PLL-g-PEG (Scheme 1). Particular attention is



Scheme 1. Surface functionalization with brush-forming graft copolymers of different side-chain compositions.

paid to the structural characteristics of the chemically different brush layers, their hydration, as well as their biopassivity and nanotribological properties, which were analyzed by a combination of surface-sensitive techniques, including variable angle spectroscopic ellipsometry (VASE), quartz crystal microbalance with dissipation (QCM-D), and atomic force microscopy (AFM)-based methods. All of these characteristics are extremely relevant when such brush films are applied on medical devices and implants that require both surface biopassivity and lubrication.

All of the graft copolymers (PLL-g-X) employed for surface functionalization feature side chains with a degree of polymerization (DP) of approximately 100, and side-chain densities on the PLL "backbone" of approximately 0.3 chains per lysine unit (X/Lys in Table 1). This particular value of X/Lys was chosen to guarantee a relatively high concentration of positively charged ammonium groups on the PLL, which drive the surface assembly, as well as a high enough side-chain loading to enable the formation of a uniform and dense brush film.[9]

VASE gave similar dry thicknesses (T_{dry} in Table 1) for all of the graft-copolymer films, with values between 1.3 and 1.5 nm (see the Supporting Information for details). The T_{dry} values were used to estimate the surface grafting density (s) for each brush type, the distance between grafting points (L), and the degree of chain overlap at the surface ($L/2R_g$). PMOXA, PEOXA, and PMOZI brushes all gave similar values of s, ranging from 0.07 to 0.10 chainsnm@^{2}, whereas PEG grafts featured a slightly higher surface density of 0.16 chainsnm@^{2}, presumably owing to the lower molar mass and molecular dimensions of PEG (Mw &5 kDaDP)(withMw rangingrespect

to PAOXAs and PMOZI with comparable from 9 to 11 kDa), which enabled the assembly of a higher graft-copolymer concentration at the surface. Despite the different values of s, the assemblies showed a similar degree of chain overlap, in all cases <1, suggesting analogous brush configuration and morphology irrespective of the graft compositions.

The combination of QCM-D and VASE data allowed us to further compare the hydration properties of the different brushes. As highlighted in Table 1, PMOXA and PMOZI brushes showed the highest concentrations of water molecules per monomer, respectively. The lower hydrophilicity of PEG and PMOZI brushes compared to PMOXA analogues was confirmed by water contact angle (CA) measurements, which showed significantly higher values for the advancing and receding CA for the two former films. Interestingly, the markedly hydrophilic character of PMOZI brushes is in agreement with the solution properties of this polymer, which, while isomeric with PEOXA, does not display a lower critical solution temperature (LCST), in a similar way to PMOXA.[34] Hence, the composition of the side groups appears to be the predominant factor determining polymer hydration, rather than the chemical nature of the main chain.[34,36]

PEOXA grafts showed the most hydrophobic character, as indicated by their limited swelling in water and relatively high values of advancing and receding CA.

Table 1: Characterization of PLL-g-X films by VASE, QCM-D, and CA (see the Supporting Information for details).

PLL-g-X	X/Lys	T_{dry} [nm]	σ [X nm^{-2}]	L [nm]	$L/2R_g$	T_{wet} [nm]	H_2O/monomer	Contact angle adv./rec. [°]
PLL-g-PMOXA	0.31	1.3 ± 0.1	0.09	3.6	0.37	8.9 ± 1.7	26 ± 3	10/6
PLL-g-PEOXA	0.33	1.3 ± 0.2	0.07	4.1	0.39	5.1 ± 1.2	14 ± 4	35/26
PLL-g-PMOZI	0.32	1.5 ± 0.1	0.10	3.3	0.35	9.2 ± 1.9	25 ± 3	13/8
PLL-g-PEG	0.34	1.3 ± 0.1	0.16	2.6	0.47	9.9 ± 1.3	19 ± 2	30/18

This behavior was further confirmed by analyzing the adhesive properties of the layers by AFM. As reported in

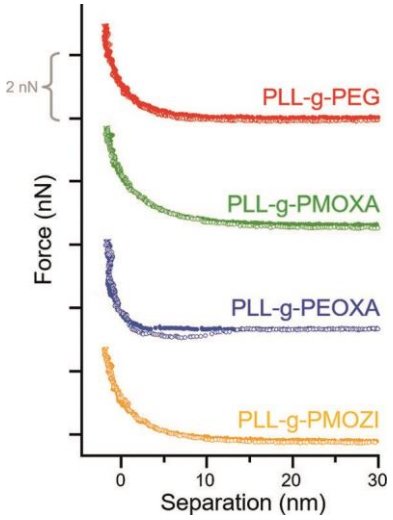


Figure 1. Force versus separation (FS) profiles recorded by AFM on the different brush layers.

Figure 1, PMOXA, PMOZI, and PEG brushes displayed marked repulsive interactions with the AFM colloidal silica probe, with the approaching and retracting profiles nearly overlapping with each other. In contrast, the force versus separation (FS) profile recorded on PEOXA-based films suggested the presence of attractive van der Waals forces between the silica colloid and the amphiphilic PEOXA chains. Adhesive interactions between the probe and the amphiphilic film were witnessed by a typical "jump-in" along the approaching curve, and the occurrence of a significant adhesion force was recorded along the retracting profile.

The physicochemical characteristics of the different brushes were mirrored by the varied resistance towards protein adsorption from solution. We specifically tested the biopassivity of PLL-g-X films, alternatively subjecting them to 10% human (HS) and fetal bovine serum (FBS) for 1 hour, and subsequently measuring the amount of adsorbed proteins by VASE. All of the brushes significantly reduced the amount of adsorbed protein both from HS and FBS compared to the bare SiO_2 surface (Figure 2). However, PEOXA brushes were the least antifouling layers, with a 91 and 85% reduction of physisorbed serum from HS and FBS, respectively. PEG and PMOXA brushes performed similarly, reducing protein contamination by more than 90%, in agreement with a previous comparative study performed in our group.[37] Surprisingly, PMOZI brushes nearly quantitatively hindered protein adsorption from both HS and FBS, reaching 97 and 96% of adsorbed-serum reduction, respectively.

The resistance towards protein fouling correlated directly with brush hydrophilicity. As shown in Figure 2c, the amount of adsorbed proteins decreased with an increase in the concentration of water molecules per monomer unit, with the most hydrophilic brushes (PMOXA and PMOZI) producing the best antifouling layers. However, the nearly quantitative resistance towards protein contamination displayed by PMOZI brushes could not be solely explained by polymer hydration, which was similar to that recorded for PMOXA grafts. We believe that the higher flexibility of PMOZI chains

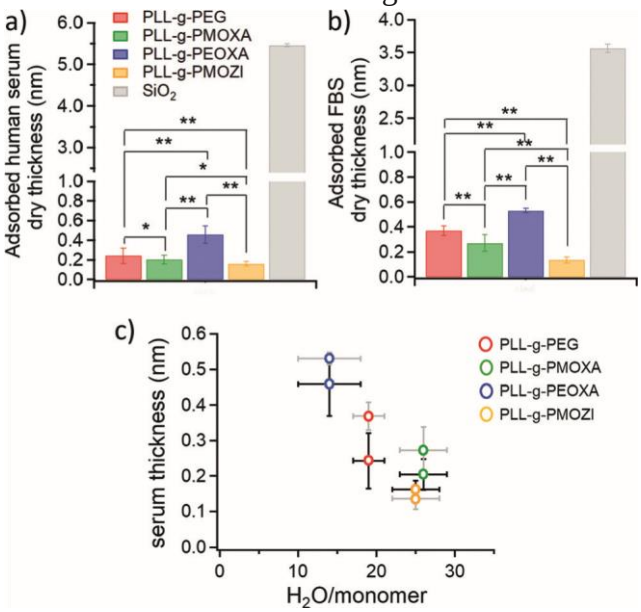


Figure 2. Protein adsorption from a) 10% HS and b) 10% FBS on PLLg-X films measured by VASE after 1 hour of exposure. c) Amount of adsorbed proteins as a function of polymer brush hydration (H_2O/ monomer). (*) $p<0.05$; (**) $p<0.01$.

with respect to its PAOXA counterparts, which results from the additional methylene group in the repeating unit along the PMOZI main chain, generates brushes that provide a more efficient entropic barrier towards approaching biomolecules. In support of this hypothesis, bulk PMOZI shows a glass transition (T_g) below ambient temperature,[33] which is significantly lower than the T_g of the isomeric PEOXA (T_g

&60As8C).brush-forming graft copolymers can be easily applied for the functionalization of implants,[14] their integration within a surrounding tissue environment was further evaluated by testing the adhesion of bovine chondrocytes. In particular, preventing unspecific cell adhesion on these brush coatings would later allow us to introduce well-determined functionalities/peptide sequences that direct cell settlement and proliferation.[11]

After 24 hours of incubation, PLL-g-PMOZI films had no cells attached, while PLL-g-PMOXA, PLL-g-PEG, and PLLg-PEOXA displayed the adhesion of 15, 20, and 60% of cells, respectively, when compared to bare SiO_2, which was chosen as a positive control (Figure 3a). In a similar way, PLL-gPMOZI quantitatively prevented the unspecific settlement of cells without complementing the culture medium with 10% FBS (Figure 3b), while PLL-g-PMOXA, PLL-g-PEG, and PLL-g-PEOXA analogues showed 2, 5, and 25% of adhered cells, respectively.

As with the biopassive character towards serum proteins, the combination of high hydration and chain flexibility by PMOZI brushes produced films that fully hindered cell adhesion (Figure 3c). It is also relevant that these unique antifouling properties were not due to any cytotoxic character of the PMOZI side chains, as confirmed by biocompatibility tests (Figure S5).

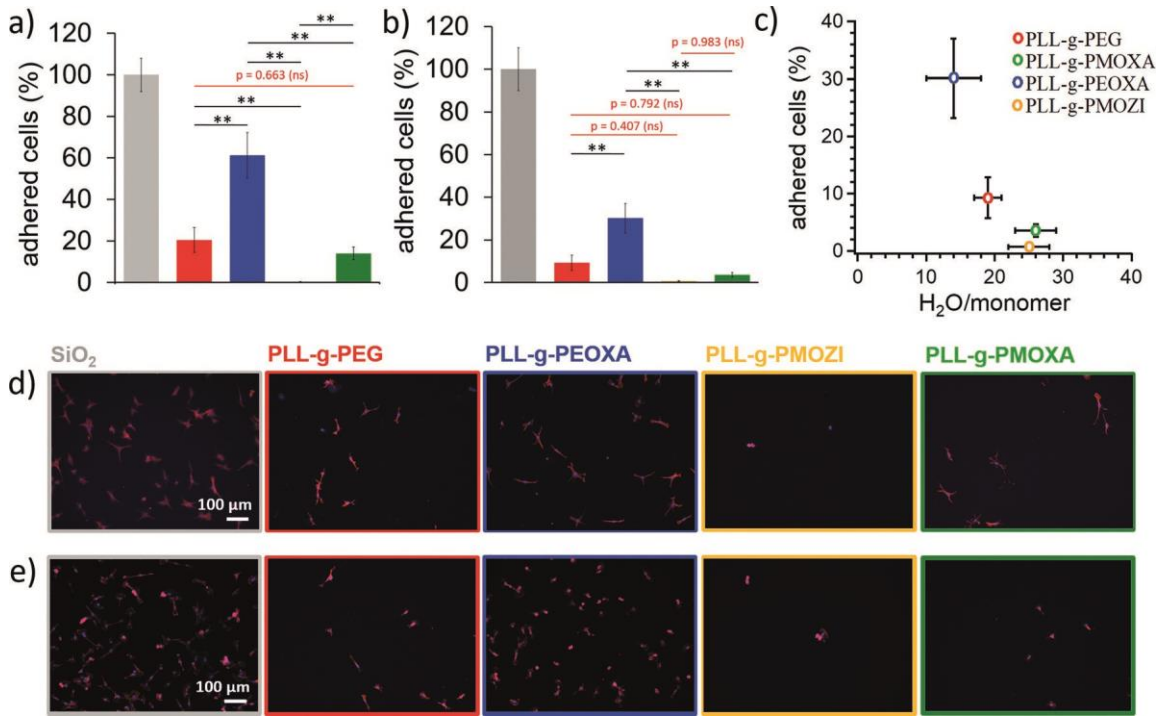


Figure 3. Bovine chondrocyte adhesion on the different PLL-g-X films after 24 hours of culture, with (a) and without (b) complemented 10% FBS. In (c), the percentage of adhered cells is correlated to brush hydration (H_2O/monomer). Representative immunofluorescence micrographs highlighting chondrocytes adhered on the different films are reported in (d) (without 10% FBS) and (e) (with 10% FBS).

Besides the resistance towards unspecific protein and cell adhesion, brush lubrication can be a fundamental requirement when these coatings are applied on the exposed surface of medical devices.

The nanotribological properties of PLL-g-X films were assessed by lateral force microscopy (LFM), recording friction force versus applied load profiles (FfL) on the different brush films.[38,39] As displayed in Figure 4, PEOXA

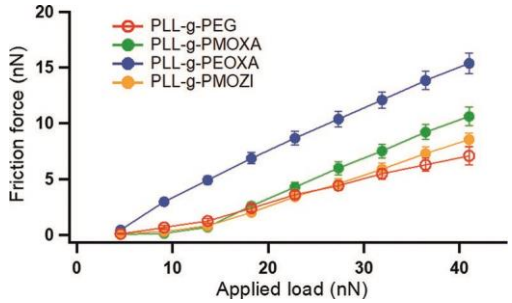


Figure 4. FfL profiles recorded by LFM on different brush films.

brushes showed the highest friction among the different films studied, probably because of their limited hydration and amphiphilic character. In contrast, an improvement in the lubrication properties was found for PMOXA, PMOZI, and PEG brushes, with the latter two brush types displaying the lowest friction.

These results corroborated the direct correlation between biopassivity and lubrication properties, which was previously found for different brush chemistries and structures,[40–42] with both these two characteristics being determined by brush surface density and polymer hydration. However, the lowest friction values recorded for PEG and PMOZI brushes suggested that when comparing the nanotribological properties of hydrophilic polymer grafts, those featuring higher chain flexibility are slightly more lubricious than more rigid brushes. In agreement with this assumption, the slope of the FfL profiles progressively decreased with the T_g of the polymer, which was 78, 16, and @358C for PMOXA,

PMOZI,[33] and PEG,[43] respectively.

In summary, a comparative analysis of PAOXA, PMOZI, and PEG brushes, formed on SiO_2 surfaces by graft-copolymer assembly, has highlighted how polymer hydration and flexibility determine the performance of the brush layers as lubricious biointerfaces. PAOXA brushes can match the biopassivity and frictional properties of PEG analogues, and outperform the attractive properties of these latter films in the case of the most hydrophilic PMOXA. The presence of an additional methylene group within the polymer-repeating unit, as in the case of PMOZI brushes, leaves their hydration capabilities unaltered in comparison to PMOXA analogues, and substantially improves them with respect to the isomeric PEOXA grafts.

The combination of high hydration and enhanced chain flexibility, guaranteed by longer propyl segments spacing the amide moieties, significantly reduces friction and generates an exceptional enthalpic and entropic barrier against protein and cell adhesion. Moreover, while featuring an analogous composition to PAOXAs, PMOZI brushes are expected to feature a similarly improved chemical resistance towards oxidative degradation compared to PEGs, especially within physiological media.[37] Hence, PMOZI has emerged as a new polymer for the generation of brushes with unprecedented properties, significantly surpassing the state of the art, and opening up a plethora of possible applications in the modification of biomaterials.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biointerfaces · friction · polymer brushes · polyoxazolines · protein adsorption

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